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FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE QUÍMICA E BIOQUÍMICA

BIOSENSOR EVALUATION OF THE ANTIOXIDANT ACTIVITY OF WINES AND TEAS. INTERFERENCE STUDIES AND COMPARISON WITH OTHER METHODS

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DOUTORAMENTO EM BIOQUÍMICA
(Especialidade Biotecnologia)

TESE ORIENTADA POR
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2011

This thesis is dedicated in loving memory to my father, Leonel Gil.

ACKNOLEDGEMENTS

Dulce M.A. Gil is the beneficiary of the grant: SFRH/BD/34511/2006 from *Fundação para a Ciência e Tecnologia* (FCT), and cordially appreciates and acknowledges their financial support.

The work was carried out at Lisbon University' Chemical/Biochemical Department during a period of two years and seven months (2008-2011). I hereby wish to express my sincere thankfulness for having providing me with the researching facilities that enabled me to undertake my project as planned.

I would like to express my deepest gratitude and respect to my supervisor, Prof. Dr. Maria José Rebelo for her expertise supervision, continuous encouragement, exceptional ideas, valuable advice, extensive knowledge and tireless optimism that have kept me going throughout this study.

I am especially thankful to Prof. Dr. Maria Luísa Serralheiro for her help over the years; especially her knowledge and expertise, critical reading and always having time for me.

I would like also to extend my gratitude to Prof. Dr. Inês Fonseca with whom I shared delightful discussions regarding the subject of Cyclic Voltammetry.

My special thanks to Pedro Falé for guiding me in the intricacies of High Performance Liquid Chromatography.

I would like to acknowledge to Eng. António Graça from Research & Development Department at SOGRAPE VINHOS S.A. for kindly have offered samples of Portuguese wine which were the subject of some of the experiment undertaken during this research study.

My sincere appreciation to all staff at Lisbon University' Chemical/Biochemical Department laboratories for their assistance.

My heart felt thanks to all my friends for their help and support.

I devote my deepest gratitude to my sister and my mother for their interest, unlimited love, encouragement and support.

This work was financially supported by *Fundação para a Ciência e Tecnologia* (FCT).

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ABSTRACT

Polyphenolic compounds are a complex group of substances commonly found in fruit, vegetables and their products, which have gained enormous relevance in the analytical chemistry field in the recent years, largely due to the concomitant health benefits associated with their antioxidant activity. Therefore, it is of great interest to evaluate the antioxidant activity of natural foodstuff and beverage related to its polyphenolic content. Thus, there is an increase demand for highly sensitive and selective analytical method for the determination of phenolics. Biosensors offer advantages as alternatives to conventional methods due to their inherent specificity, simplicity and quick response.

An amperometric biosensor based on laccase, from *Tramates versicolor* (TvLac) developed by our group was used to determine the total polyphenolic content in wine and green tea/herbal infusions samples. The purpose of this work comprised the assessment of the antioxidant activity of those samples in relation to their polyphenolic content using a laccase based biosensor. Interference studies on polyphenolic amperometric biosensing were performed for metabissulfite and gallic acid (GA).

In order to investigate these subjects, the following analytical methods were performed: Determination of bioelectrochemical polyphenolic index (BPI) using the laccase-based biosensor; Determination of total phenolic content (TPC) using Folin–Ciocalteu method; Determination of total antioxidant activity (TAA) using ABTS assay; Cyclic voltammetry; HPLC – DAD analysis; Spectroscopic and TvLac activity/inhibition assays.

The results obtained showed that the linear relationship between total antioxidant activity and bioelectrochemical polyphenol index determined by our laccase biosensor enabled the calculation of the TEAC (Trolox Equivalent Antioxidant Capacity) of a wine and tea, the most widely consumed beverages worldwide, from its bioelectrochemical polyphenol index. It was found that immobilised laccase was less inhibited by metabissulfite than free laccase. A confirmation of the influence of metabisulfite on Folin-Ciocalteu measurements was also

undertaken. The results have indicated that GA presents an inhibitory behaviour on TvLac activity in a concentration-dependent manner. The GA' interference on polyphenolic amperometric biosensing is the result of the combination of two factors: on one hand, we have the inhibitory enzymatic effect, and on the other, the reaction of GA' oxidation products with the o-quinones obtained by the enzymatic oxidation of caffeic acid. Both gave rise to the amperometric signal decreasing effect. The very strong correlation (r^2 equaled 0.9949) between BPI/TPC obtained for herbal infusions, allowed us to conclude that the laccase-based biosensor, used in this research, provided a valuable tool to obtain a valid estimation of the classical Folin-Ciocalteu index, in an uncomplicated and fast way.

Keywords: Laccase-biosensor; Phenolic compounds; Antioxidant activity; Wines; Teas; Interferences.

RESUMO

Os polifenóis constituem um complexo grupo de substâncias comummente encontradas em frutas, vegetais e seus produtos. A importância da pesquisa de compostos fenólicos em alimentos/bebidas deve-se ao facto de muitos destes compostos apresentarem propriedades antioxidantes. A acção dos antioxidantes no combate ao stress oxidativo tem sido objecto de estudo em diversos trabalhos que demonstram a importância destes compostos na prevenção de doenças associadas ao envelhecimento, diminuindo o risco de doenças cardiovasculares e o aparecimento de cancro. A actividade antioxidante dos compostos fenólicos depende basicamente de algumas das suas propriedades intrínsecas, tais como, o potencial de redução, a capacidase de quelar metais, e da possibilidade de captar/sequestrar radicais livres.

É de grande interesse avaliar a actividade antioxidante de alimentos naturais e de bebidas relacionada a seus constituintes polifenólicos. Para esse efeito, uma série de diferentes métodos analíticos encontram-se disponíveis, tais como GC, HPLC e métodos espectrofotométricos. No entanto, estas técnicas são dispendiosas, consomem reagentes e tempo. O método de Folin-Ciocalteu é uma das abordagens mais utilizadas para a determinação espectrofotométrica do conteúdo total de compostos fenólicos em alimentos. No entanto, este método produz uma sobreestimativa do teor de polifenóis totais (TPC), devido à sua baixa especificidade, uma vez que o reagente Folin-Ciocalteau reage com outras substâncias redutoras não fenólicas presentes na amostra.

Consequentemente, verifica-se um aumento na procura de métodos analíticos mais sensíveis e selectivos para a determinação de compostos fenólicos. Assim sendo, os biossensores oferecem grandes vantagens como alternativa aos métodos convencionais devido a serem simples, sensíveis, confiáveis e de resposta rápida, necessitando de instrumentação de baixo custo, operam em condições em que não é necessário um pré-tratamento da amostra, e permitem efectuar determinações numa ampla faixa de concentração.

Um biossensor pode ser definido como um sensor que combina a actividade selectiva de um elemento biológico sensível ao analito de interesse, ligado a um transdutor que converte o sinal biológico em um sinal eléctrico proporcional à concentração do analito. Os biossensores eletroquímicos podem ser divididos em: amperométricos, potenciométricos e condutimétricos.

O princípio de funcionamento dos biossensores amperométricos é caracterizado pela medida da corrente produzida por uma reacção química entre espécies electroactivas. Esta reacção ocorre num potencial determinado, e a corrente gerada está relacionada com a espécie em solução. Assim estes biossensores dependem tipicamente de um sistema biológico que converta cataliticamente analitos inactivos electroquimicamente em produtos que possam ser oxidados ou reduzidos num eléctrodo de trabalho, o qual é mantido a um determinado potencial vs um eléctrodo de referência. A corrente produzida pela reacção redox é linearmente proporcional à concentração do produto eletroactivo, a qual é proporcional ao analito (substrato da enzima) não eletroactivo.

Na realização do trabalho experimental foi empregue um biossensor amperométrico baseado no enzima lacase de *Trametes Versicolor* (EC. 1.10.3.2) (TvLac) imobilizado numa membrana de polietersulfona. Lacase é um oxidase com múltiplos centros de cobre que catalisam a oxidação de vários compostos aromáticos como é o caso dos polifenóis, a quinonas e/ou a espécies radicalares. Nesta reacção, o oxigénio é reduzido directamente a água, sem a formação de peróxido de hidrogénio. Então, no sistema biossensor, a lacase catalisa a oxidação de polifenóis às o-quinonas correspondentes, que são reduzidas ao polifenol inicial, no eléctrodo. A corrente resultante é utilizada como resposta analítica.

O trabalho experimental realizado nesta tese objectivou-se na avaliação de actividade antioxidante de vinhos Portugueses (branco, rosé e tinto) e de chás/infusões de ervas, em relação ao seu conteúdo fenólico total usando o biossensor à base de lacase. O estudo de interferências no desempenho do biossensor foi realizado para o metabissulfito e para o ácido gálico (GA).

A investigação dos referidos objectivos envolveu fundamentalmente a realização dos seguintes métodos analíticos:

- Determinação do índice bioelectroquímico polifenólico (BPI) ,em equivalentes de ácido caféico, usando o biossensor baseado em lacase;
- Determinação do conteúdo total polifenólico (TPC), usando o método de Folin-Ciocalteu;
- Determinação da actividade antioxidante total (TAA), usando o método de *ABTS ou* TEAC (trolox equivalent antioxidant activity);
- Voltametria cíclica;
- Análises por HPLC-DAD;
- Determinação de actividade/inibição enzimática de TvLac.

Os resultados obtidos nos estudos desenvolvidos mostraram que:

- Foi observada uma boa correlação entre as propriedades antioxidantes (TAA) dos vinhos estudados (9 tintos, 5 brancos e 3 vinhos rosés), determinado pelo ensaio ABTS e o BPI em pH 3,5, com o biossensor, tomando ácido caféico como a solução de referência.

- A relação linear entre TAA e BPI permitiu o cálculo da actividade antioxidante de vinhos tintos, rosé e branco, a partir do seu conteúdo em equivalentes de ácido caféico, avaliado pelo biossensor .

- A metodologia empregue no biosensor baseado em lacase apresenta vantagens em relação a outras. Na verdade o bioeléctrodo é muito simples de preparar, com uma rápida imobilização do enzima na membrana polietersulfona. Além disso, as medições da solução de referência e da amostra de vinho foram realizadas em pH 3,5, que é um valor próximo do pH médio de vinhos.

- O metabissulfito constituiu interferência nas medições realizadas com os biossensores à base de TvLac e também influenciou os resultados obtidos pelo método espectrofotométrico de Folin-Ciocalteu.

- Relativamente ao GA foi observado um efeito sinergético negativo na resposta do biossensor, a uma mistura de polifenóis contendo o GA. Os resultados indicaram que o GA apresenta comportamento inibitório sobre a actividade do TvLac, dependente da concentração. De facto, os resultados apresentados neste trabalho demonstraram que a presença de GA diminui a actividade enzimática do TvLac em solução e imobilizado. Além disso, foi demonstrado que a oxidação de GA e dos produtos subsequentes (o-quinonas), interferiram com o ácido caféico (CA) e os produtos da oxidação de CA, contribuindo, deste modo, para a redução efectiva da resposta do biosssensor, devido ao desaparecimento dos produtos finais enzimaticamente oxidados.

A correlação muito forte (r^2 = 0,9949) entre o BPI / TPC obtida para infusões de ervas permitiu concluir que o biossensor, utilizado neste trabalho, apresenta-se como uma útil ferramenta analítica para obtenção de uma estimativa válida do clássico índice de Folin-Ciocalteu, de uma forma simples e rápida.

Assim sendo, recomenda-se de que os resultados obtidos com os biossensores à base de lacase e com o método espectrofotométrico de Folin-Ciocalteu devem ser relatados como "equivalente de ácido caféico". Trabalho futuro deverá ser realizado a fim de quantificar e/ou evitar o efeito das substâncias interferentes referidas.

Palavras-chave: Biossensor à base de lacase; Polifenóis; Actividade antioxidante; Vinhos; Chás; Interferências.

LIST OF ABBREVIATIONS AND SYMBOLS

A	absorbance
Å	Angstron
$A^{\bullet}$	antioxidant free radical
ABTS	2,2'- azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
$ABTS^{\bullet+}$	2,2´-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation
Ag/AgCl	silver/silver chloride
AH	antioxidant
AU	Absorbance units
BPI	bioelectrochemical polyphenolic index
CA	caffeic acid
CAE	caffeic acid equivalents
CV	cyclic voltammetry
Da	Dalton
DET	direct electron transfer
DPPH	2,2-diphenyl-1-picrylhydrazyl assay
e^-	electron
E_0'	formal potential
E_{ap}	applied potential
$E_{p,a}$	anodic peak potential
$E_{p,c}$	cathodic peak potential
ET	electron transfer
FC	Folin–Ciocalteu
FCR	Folin–Ciocalteu reagent
FRAP	ferric reducing antioxidant power
GA	gallic acid
GAE	gallic acid equivalents
GC	gas chromatography

GCE	glassy carbon electrode
H^+	Hydrogen ion
H_2O_2	hydrogen peroxide
H_2SO_3	sulfurous acid
HAT	hydrogen atom transfer
HPLC-DAD	high performance liquid chromatography - diode array detector
HSO_3^-	sulfite
I	inhibition
IC_{50}	half-maximal inhibitory concentration
$I_{p,c}$	cathodic peak current
$I_{p,a}$	anodic peak current
$K_2S_2O_8$	potassium persulfate
K_m	Michaelis-Menten constant
L	litre
$L^\bullet$	lipid radicals
$LO^\bullet$	lipid alkoxyl radicals
LOD	limit of detection
$LOO^\bullet$	lipid peroxyl radicals
M	molar
mg	milligram
min	minute
mL	millilitre
mm	millimeter
mM	millimolar
mV	millivolt
n	number of electrons
nA	nanoampere

$Na_2S_2O_5$	sodium metabisulfite
$NaHSO_3$	sodium bisulfite
nm	nanometre
$O_2^{\bullet -}$	superoxide anion
$OH^{\bullet}$	hydroxyl radical
ORAC	oxygen radical absorbance capacity
PES	polyethersulfone
PO	phenol oxidase
PPO	polyphenol oxidase
$R^{\bullet}$	Free radicals
RNS	reactive nitrogen species
ROS	reactive oxygen species
RP-HPLC	reversed-phase high performance liquid chromatography
SCE	saturated calomel electrode
SO_2	sulfur dioxide
SO_3^{2-}	sulphite ion
TAA	total antioxidant activity
TEAC	trolox equivalent antioxidant capacity
TPC	total phenolic content
TRAP	total peroxyl radical-trapping antioxidant parameter
TvLac	laccase from *Trametes versicolor*
%	percent
µg	microgram
µL	microlitre
µM	micromolar
U	enzyme unit
V	Volt

v/v	volume per volume
V_{max}	maximum reaction rate
w/v	weight per volume
ε	molar absorption coefficient
λ_{max}	maximum wavelength
UV-vis	ultraviolet-visible

CHAPTER 1

INTRODUCTION

1.1. General introduction

In recent years, there has been a growing interest in obtaining biologically active compounds from natural sources. Antioxidants act preventing the oxidative damage to biomolecules caused by free radicals, and are thought to aid in the prevention of many health problems, such as heart disease, cancer, and inflammatory diseases. The protective effects that diets rich in fruit, vegetables and their products, have on those pathogies, are being partly attributed to their antioxidant content. Wine and tea are a potential natural antioxidant source due to their rich phenolic content. Thus, the measurement of antioxidants in consumer products as wine and teas is of relevant importance.

The determination of phenolic compounds is regarded to be of great importance in food quality characterisation. For that purpose, there are a number of different analytical methods available, such as GC, HPLC and spectrophotometric methods. However, these techniques are expensive, reagent and time consuming. Moreover, the Folin-Ciocalteu method is one of the most accepted approaches for the spectrophotometric determination of the total content of food phenolics. Nevertheless, this spectrophotometric approach yields an overestimation of the total polyphenolic content (TPC) due to its poor specificity, since Folin-Ciocalteau reagent reacts with other different reducing nonphenolic substances.

Biosensors have been proposed as an alternative and efficient analytical tool for the determination of polyphenolic compounds, exhibiting advantages such as the minimal preparation of the sample, selectivity, sensitivity, reproducibility, rapid time of response, low cost, miniaturization, easy automation, time saving, simplicity of operation and manufacturing. Thus, the development of biosensors for the polyphenolic determination of teas and wines is a great ongoing challenge.

Biosensors can be defined as an analytical tool or system consisting of an immobilized biological material in intimate contact with a suitable transducer. Biological recognition elements are the major selective element in a biosensor system. They can be organisms, tissues,

organelles, enzymes, antibodies and nucleic acids. The type of the biological recognition element determines the degree of selectivity or specificity of the biosensor. The transducer element in a biosensor system converts the biological signal into an electrical one.

Biosensors utilise biological components for the detection of biologically relevant analytes. Enzymes are biological elements extremely specific and selective for the substrates which they interact with. Laccase is an enzyme that is being object of intensive study in the phenolic oxidation research field. It is an oxidoreductase (polyphenoloxidases) able to catalyze the oxidation of various aromatic compounds, particularly phenolic compounds with the concomitant reduction of oxygen to water. It displays a broad specificity for the reducing substrates including mono-phenols, di-phenols, polyphenols, amino phenols, and aromatic diamines. The broad specificity for the phenolic substrates enables laccase to be developed as a biosensor for the determination of total phenolics.

The amperometric biosensors are the most commonly used electrochemical sensors for analysis of food and beverages. Amperometric biosensors utilize the analytical capability of amperometry together with the specificity of enzymes or other bioelements resulting in sensors with high sensitivity and selectivity. An amperometric biosensor may be more attractive than other biosensor systems due to its promising properties such as high sensitivity and a wide linear range. Amperometric biosensors are based on the measurement of the current resulting from the oxidation or reduction of electroactive species. In amperometry, the resulting current is correlated to the bulk analyte concentration of the electroactive species.

A large number of amperometric biosensors have been seen and proposed as being attractive alternative analytical methods for the quantification of phenolic compounds due to their inherent specificity, simplicity and fast response, being most of them based on the immobilisation of laccase on different electrode supports.

1.2. Objectives and Goals

An amperometric biosensor containing immobilised laccase from *Trametes versicolor (TvLac)* was used for the quantification of phenolic compounds. The biosensor parameters and optimisation of suitable analytical conditions for measurements was previously performed by our group.

Optimizing the applicability of biosensors to real samples and comparing the corresponding measured values to those of other methods, are some of the greatest challenges in present biosensor research.

Assuming this challenge, we tried to correlate our bioelectrochemical polyphenolic index (BPI), as caffeic acid equivalent of different Portuguese wines (white, rosé and red samples) with the Folin–Ciocalteu index, which, in spite of several disadvantages and limitations, is one of the most accepted approaches for the determination of the total polyphenol index in food chemistry. The other goal was to correlate the biosensor data with the wine antioxidant capacity using the TEAC (Trolox Equivalent Antioxidant Capacity) methodology.

As it is well known, the applicability of electrochemical biosensors to the analysis of antioxidant compounds, including polyphenols, is promising and there is a growing interest in the development of such devices. However, it is reckoned that further work will have to be undertaken to avoid and/or take into account the interference problem.

In fact, interferences from substances in real matrices occur, whatever method is used. Though many authors do not perform studies on the interference of sulfites and sulphurous species, which are used as food preservatives, also in wine, it is most probable that their measurements are affected by some contribution from them.

Electrochemical reactions and adsorption of sulphurous species on electrode surfaces have been studied for a long time and have been a matter of concern. Despite that, those effects are not completely clarified and, thus, more studies have been done and need to be done.

Actually, an upsurge of interest in their study appeared in recent years, due to increasing concerns regarding allergic reactions caused by sulfites added in food, though the actual cause of allergic reactions is a matter of controversy**.** On the other hand, polyphenoloxidases are inhibited by sulfite, and consequently, they seriously compromise the biosensor responses.

The present work was, thus, part of a continuous effort to get a better understanding of the interference that sulphurous species have whenever polyphenol measurement is being undertaken.

In order to meet this goal, that is, to investigate the possibility of the sulfite interference on biosensor response, the following tasks were performed:

- Response of the sensor and the laccase-based biosensor to metabisulfite containing solutions;
- Cyclic voltammetry analysis;
- Studies of the influence of metabisulfite on the activity of the free or immobilised enzyme laccase;
- Measurement of the metabisulfite interference on the determination of phenolic compounds by Folin–Ciocalteu method.

Some research groups on amperometric laccase-based biosensors mentioned to have obtained a low sensitivity for gallic acid (GA). It is known that TvLac belongs to the well studied laccases, however to our knowledge, there are no studies in the literature regarding TvLac inhibition by GA. Therefore, the purpose of the present work was to investigate the possibility of GA interference on polyphenolic amperometric biosensing using TvLac. Thus, GA' inhibitory effect on TvLac activity was investigated on the oxidation of caffeic acid (CA) by free Tvlac and its

immobilised form on modified polyethersulfone membrane (PES/TvLac), using comparative spectrophotometric and amperometric biosensor detection methods. Subsequently, the electrochemical behavior of CA and GA was also studied by cyclic voltammetry, in order to verify a possible interfering effect on the amperometric sensor system.

As is well known, gallic acid present in wine varies in a wide range of concentration, and in addition, the wine represents a sample with complex matrix. So, in order to investigate the inhibitory effect of gallic acid, samples with less complex matrices than wine are required, and therefore, green tea and several herbal infusions samples were selected and used in our experimental work.

One of the aims of this part of the work was to correlate the caffeic acid equivalent of different herbal infusions and green tea using our laccase-based biosensor with the Folin-Ciocalteu index. The other goal was to correlate the biosensor data with the herbal infusion and green tea antioxidant capacity using the TEAC (Trolox Equivalent Antioxidant Capacity) methodology. Thus, the present work has investigated this potential interference in laccase-based biosensor response using samples of herbal infusions and green tea. In order to meet this *desideratum*, the following tasks were performed:

- Recovery studies;
- Analysis of phenolic compounds in herbal infusions and green tea using HPLC-DAD;
- Studies of the influence of gallic acid on the laccase' enzymatic activity.

1.3. Thesis overview

This thesis is presented as a compilation of 4 chapters.

Chapter 1 Introduction

The first chapter introduces the thesis work.

Chapter 2 Literature review

The second chapter reviews the current state of art on biosensor evaluation of the antioxidant activity of wines and teas.

Chapter 3 Articles

The third chapter is based on articles already published, revised, and submitted to international scientific journals, which reflect all the experimental work carried out during this thesis.

Chapter 4 General conclusions

Finally, the fourth chapter allocates the final conclusions and future research.

CHAPTER 2

LITERATURE REVIEW

2.1. Free radicals

The oxidative metabolism is essential for the survival of the cell, the production of free radicals being one of the side effects of this process (Antolovich et al., 2002). A free radical is any chemical species with at least one or more unpaired electrons in the outermost shell, being capable of independent existence. Because of their avidity to accept electrons from other molecules, and thereby generate more stable species, free radicals can modify the structure and/or function of biomolecules (lipids, proteins/enzymes, carbohydrates, DNA), interfering with normal cell biology (Droge, 2002; Valko *et al*., 2004; Bahorun *et al*., 2006; Halliwell and Gutteridge, 2007; Valko *et al*., 2007).

The most important free radicals in biological systems are those derived from oxygen, more commonly known as reactive oxygen species (ROS), although there are also reactive species derived from nitrogen (RNS) or sulphur (RSS). ROS include free radicals such as hydroxyl radical ($OH^{\bullet}$), superoxide anion ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), which is not a free radical but is considered as a ROS involved in the production of other free radicals. The hydroxyl radical is considered the most reactive radical known, and will react with the first available biomolecule that it meets. It can be produced *via* the Fenton reaction, $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$, first described in 1894 (Martin *et al*., 2003, Halliwell, 2007; Vermerris and Nicholson, 2006; Perron and Brumaghim, 2009).

Free radicals are continuously produced during normal physiologic events in the cells (*e.g*., cytoplasm, mitochondria or the membrane), as by-products through electron transport chains during normal aerobic (oxidative) metabolism, immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, cancer, aging. Free radicals are also generated by exogenous sources, through exposure to factors such as UV, gamma, X-ray radiation, cigarette smoke, environmental pollutants, lipid oxidation products in foods, and medication/drugs (Droge, 2002; Valko *et al.,* 2007; Willcox and Catignani, 2004; Pacher *et al.* 2007; Genestra, 2007; Halliwell, 2007; Young and Woodside, 2001; Valko *et al.,* 2006, Valko *et al*., 2005; Parthasarathy *et al*., 1999; Cadenas and Davies, 2000) (Table 1). After penetration

into the body by different routes, the exogenous compounds are decomposed or metabolised into free radicals.

Table 1. Partial list of endogenous and exogenous sources of free radical generation.

Source	Mechanism
Endogenous	
Mitochondrial electron transport	Leakage of superoxide due to inefficient reduction of oxygen
Transition metal ions	Copper and iron facilitate hydroxyl radical formation
Inflammation	Free radicals released by activated phagocytes
Enzymes e.g. Xanthine Oxidase	Release superoxide during reperfusion of ischemic tissues
Exogenous	
Drug metabolism	Free radical intermediates created during metabolism
Cigarette smoke	Gas phase rich in free radicals
Radiation	X-rays, UV light

All of the macromolecules found within the body are susceptible to oxidative modification by free radicals, including lipids, DNA and protein. Lipid peroxidation is probably the most extensively investigated free-radical induced process (Halliwell and Gutteridge, 2007). Lipid oxidation is a complex process often involving multiple factors and occurs in almost every biological system. The lipid component of biological membranes is highly susceptible to oxidation and may undergo a rapid and destructive chain peroxidation process (Frankel, 1998). Lipid oxidation proceeds via three different pathways; (a) non-enzymatic free radical-mediated chain reaction, (b) non-enzymatic, non-radical photo-oxidation and, (c) enzymatic reaction. Pathway (a) is the classical free radical route, and occurs in a four-step mechanism: initiation, propagation, branching and termination (Frankel, 1998; Antolovich *et al.,* 2002).

Initiation:

$$LH + R^{\bullet} \rightarrow L^{\bullet} + RH$$

Propagation:

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH$$

Branching:

$$LOOH + LH \rightarrow LO^{\bullet} + HO^{\bullet}$$

$$2\ LOOH \rightarrow LOO^{\bullet} + LO^{\bullet} + H_2O$$

$$LOOH + M^{n+} + H^{+} \rightarrow LO^{\bullet} + M^{(n+1)+} + H_2O$$

$$LOOH + M^{(n+1)+} + OH^{-} \rightarrow LOO^{\bullet} + M^{n+} + H_2O$$

Termination:

$$\left.\begin{array}{l} LO^{\bullet} + LO^{\bullet} \rightarrow \\ LOO^{\bullet} + LOO^{\bullet} \rightarrow \\ LO^{\bullet} + LOO^{\bullet} \rightarrow \end{array}\right\} \text{non-radical products}$$

The initiation step, which is characterised by the initial formation of lipid radicals ($L^{\bullet}$), takes place when initiating factors such as free radicals ($R^{\bullet}$), transition metal ions, temperature and/or ionising radiation remove an electron from a stable, electron-rich species (Frankel, 1998). Oxidation via these lipid radicals is slow at first, because the concentration of radicals is low. However, once exposed to oxygen the initial lipid radicals will form lipid peroxyl radicals ($LOO^{\bullet}$) and the lipid oxidation process will rapidly increase. The propagation step of the classical pathway is defined by a rapid increase of a free radical induced lipid oxidation reaction. This step is characterised by an uncontrolled free radical chain reaction. In aerobic systems, lipid oxidation and formation of lipid peroxide radicals occur more rapidly, thereby leading to more lipid peroxide radicals available to react with unsaturated double bonds (Frankel, 1998). The breakdown of lipid hydroperoxides often involves transition metal ion catalysis, in reactions analogous to that with hydrogen peroxide, yielding lipid peroxyl and lipid alkoxyl radicals. The final step of lipid oxidation is the termination step during which the concentrations of lipid

peroxide radicals is so high that radical-radical collisions occur and form nonradical products (Frankel, 1998; Antolovich, *et al*. 2002).

ROS/RNS have well defined cell functions and play essential role in many physiological reactions, as *e.g.,* catalytic oxidation of some endogenous compounds and xenobiotics. However, they are, at higher concentrations, the cause of severe oxidative damage of cell components (Young and Woodside, 2001; Droge, 2002; Pacher *et al*. 2007; Genestra, 2007; Halliwell, 2007; Vera-Ramires *et al*., 2011).

Oxidative stress occurs when cells cannot adequately counteract the excess of free radicals formed during cellular metabolism. In other words, oxidative stress arising as a result of an imbalance between formation and neutralisation of ROS by protective mechanisms, referred to as antioxidant defence system (Halliwell and Gutteridge, 2007; Reuter *et al*., 2010, Durackova, 2010).

This imbalance leads to cellular injuries and initiate peroxidation of polyunsaturated fatty acids in biological membranes, as referred above (Frankel, 1998; Antolovich *et al*,. 2002). The tissue injury caused by ROS may include DNA damage (Halliwell, 2007; Valko *et al.*, 2004), protein damage (Bartold *et al*., 1984), and oxidation of important enzymes (Varani *et al*., 1985) in the human body. These events could consequently lead to the occurrence of various free radical related diseases: acute respiratory distress syndrome (Lichtenstern *et al.,* 2010); Alzheimer's disease (Zawia *et al*. 2009; Bolaños *et al*., 2009); cancer (Valko. *et al*., 2004; Willcox and Catignani, 2004; Valko *et al*., 2006; Valko *et al*., 2007; Halliwell, 2007; Vera-Ramirez *et al*., 2011; Reuter *et al*., 2010); cardiovascular disease (Fearon and Faux, 2009); diabetes (Rains and Jain, 2011); obesity (Atabek, 2004); Parkinson's disease (Bolaños *et al*., 2009; Shadrina *et al*, 2010).

2.2. Antioxidants

The body has several defence mechanisms to neutralise the oxidative stress through the use of antioxidation agents. Antioxidants can be produced in the cells (endogenous antioxidant) and/or supplied externally through food/supplements (exogenous antioxidants).

An antioxidant may be defined as “any substance, which present in a low concentration in comparison with the oxidised substance, delays or inhibits significantly oxidation of this substance” (Halliwell and Gutteridge, 2007). The antioxidants’ functions are to counteract the excess of free radicals, to protect the cells against their toxic effects, and to contribute to disease prevention. They may act as free radical scavengers, hydrogen donors, electron donors, peroxide decomposers, singlet oxygen quenchers, synergists, metal-chelating agents, and/or activators of antioxidative defence’ enzyme systems to suppress the radical damages in biological systems (Nickavar *et al*., 2008).

According to the antioxidant activity’ mechanism, it is possible to divide them into two classes (Niki *et al.,* 1984; Jadhav *et al.,* 1996; Antolovich, *et al*. 2002; Gramza and Korczak, 2005; Mello and Kubota, 2007):

- **Primary (chain-breaking) antioxidants or donors,** act by blocking free radicals converting them into stable products via redox reactions. As, *e.g*., phenolic compounds and tocopherols that inactived peroxides by quenching free radicals of fatty acids, the antioxidant hydrogen interrupting the reaction sequence; simultaneously the antioxidant loses its activity;
- **secondary (preventative) antioxidants or the acceptors**, are compounds that retard the rate of the chain initiation of oxidation, which protect lipids by binding air oxygen or which delay lipid oxidation in the result of processes different from breaking of autoxidation chain reactions. This may be achieved in a number of ways, such as: complexing metal ions catalyzing autoxidation-chelators (ascorbic acid); quenching singlet oxygen (β-carotene); partial regenerating of primarily antioxidants (synergistic) (ascorbic acid regenerating the vitamin E); scavenging oxygen (ascorbic acid);

decomposing peroxides and nonradical products (Maillard reaction products); absorbing UV radiation.

Primary antioxidants, AH, donate electrons to free radicals, stabilizing the radical in the process. These antioxidants are referred to as radical scavengers. Primary antioxidants when present in trace amounts can prevent oxidation by two mechanisms (Jadhav *et al.,* 1996; Antolovich, *et al.* 2002): they may either delay or inhibit the initiation step by reacting with a lipid radical (oxidised substrate) or inhibit the propagation step by reacting with peroxyl or alkoxyl:

$L^{\bullet} + AH \rightarrow LH + A^{\bullet}$

$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$

$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet}$

The antioxidant free radical ($A^{\bullet}$) may further interfere with chain propagation reactions by forming peroxy antioxidant compounds:

$A^{\bullet} + LOO^{\bullet} \rightarrow LOOA$

$A^{\bullet} + LO^{\bullet} \rightarrow LOA$

Examples of primary antioxidants are α-tocopherols and certain flavonoids.

The activation energy of the above reactions increases with increasing A-H and L-H bond dissociation energy. Therefore, the efficiency of the antioxidant increases with decreasing A-H bond strength (Antolovich, *et al.* 2002).

Chain-breaking antioxidants may occur naturally or be produced synthetically as in the case of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, tocopherol, rosemary extract and ascorbic acid (Gramza and Korczak, 2005; André *et al.,* 2010). Most of the synthetic antioxidants are of the phenolic type (Gramza and Korczak, 2005; André *et al*., 2010). These synthetic antioxidants are widely used in the food industry and are included in the human diet. However, many investigations suggest that limitations should be put in place regarding the use of synthetic antioxidants due to their toxicity (André *et al.,* 2010).

Foods contain a variety of antioxidant compounds, including enzymes (*e.g*. superoxide dismutase, glutathione peroxidase, and catalase), large molecules (*e.g*. albumin and ferritin), small molecules (*e.g*. ascorbic acid, glutathione, uric acid, carotenoids, and phenolic compounds), and some hormones such as estrogen, angiotensin, and melatonin (Prior *et al.,* 2005). Dietary antioxidants have a fundamental role in helping the endogenous physiological antioxidants (Table 2) to counteract oxidative stress.

Table 2. Endogenous physiological antioxidants (Fang *et al.,* 2002).

Antioxidant	Properties
Non-enzymatic	
Ascorbic acid (vitamin C)	Free radical scavenger; recycles vitamin E
α-tocopherol (vitamin E)	Major chain-breaking antioxidant in cell membrane
Glutathione (GSH)	Multiple roles in cellular antioxidant defence
Uric acid	Scavenger of OH radicals
α -lipoic acid	Recycles vitamin C; effective glutathione substitute
Metal ions: e. g. transferrin	Chelate metals ions responsible for fenton reactions
NO	Free radical scavenger; inhibitor of lipid peroxidation
Enzymatic	
Superoxide dismutase (SOD)	Removes $O_2^{\bullet -}$ by accelerating formation of H_2O_2.
Glutathione peroxidase (GPx)	Removes H_2O_2 and organic hydroperoxide
Catalase (CAT)	Convert H_2O_2 to water

In recent years, the use of natural antioxidants has been promoted and the literature has recognised that the replacement of synthetic antioxidants by natural ones may have several benefits. The research on natural antioxidants has been mainly focused on phenolic compounds, in particular flavonoids as potential sources of natural antioxidants. Phenolic compounds are reducing agents and their potential health-related properties have been ascribed to their powerful antioxidant abilities, which may protect the body from damaging oxidation reactions, caused by free radicals (Balasundram, *et al.* 2006, Moure, *et al*. 2001, Bonilla, *et al*. 2006).

2.3. Phenolic compounds

Phenolic compounds, constitute one of the most numerous and widely-distributed groups of substances in the plant kingdom. They are products of the secondary metabolism of plants, which are important determinants in the sensory and nutritional quality of fruits, vegetables and other plants (Tomas-Barberan *et al*., 2000; Lapornik *et al.,* 2005). Dietary polyphenols have been shown to play important roles in human health. High intake of vegetables, fruits, whole-grain and some beverages (tea, juice, wine) (Leopoldini *et al.,* 2011), which are rich in polyphenols, has been reported to prevent or delay a number of many chronic diseases including cancer, cardiovascular disease, chronic inflammation and many degenerative diseases (Everitt *et al.,* 2006). Many of the biological activities of phenolic compounds are attributed to their potent antioxidant capacities (Dumitru *et al*., 2008; Naczk and Shahidi, 2006). Phenolic compounds present an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule (phenolic acids) to that of a complex high-molecular mass polymer such as condensed tannins (Balasundram, 2006).

2.3.1. Main classes of phenolic compounds

Phenolic compounds comprise a wide variety of molecules that have a polyphenol structure (i.e. several hydroxyl groups on aromatic rings), but also molecules with one phenol ring, such as phenolic acids. Polyphenols are divided into several classes according to the number of phenol rings that they contain and to the structural elements that bind these rings to one another. More than 8000 polyphenolics, including over 4000 flavonoids have been identified, and the number is still growing (Tsao, 2010).

The main groups of phenolic compounds are: flavonoids, phenolic acids, tannins (hydrolysable and condensed), stilbenes and lignans (D'Archivio *et al.,* 2007) (Figure 1).

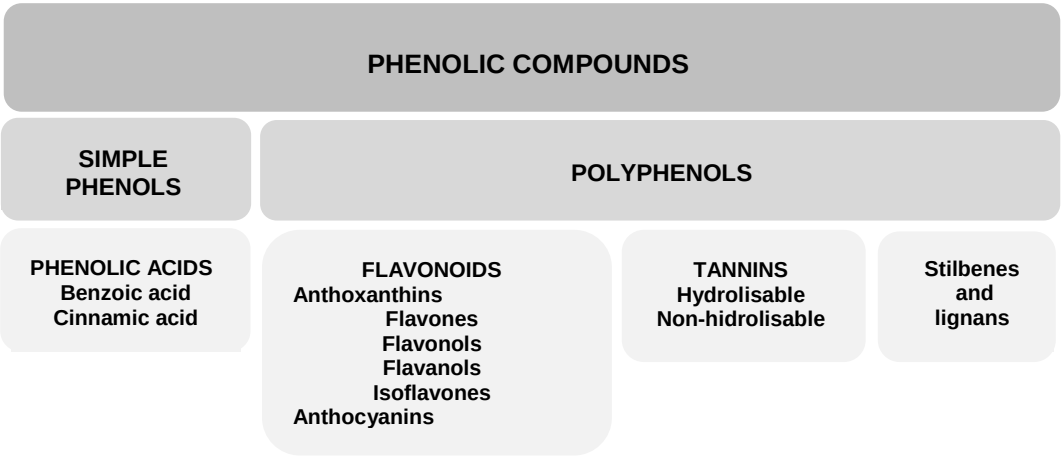


Figure 1. A simplified classification of phenolic compounds.

2.3.1.1 Flavonoids

Flavonoids are the most common and widely distributed group of plant phenolics. Flavonoids mainly exist in plants as glycosides, while aglycones (lacking sugar moieties) occur less frequently. Their common structure is that of benzo-pyrone, which has 15 carbon atoms arranged in three rings (C_6-C_3-C_6) consisting of two benzene rings (A and B), which are connected by an oxygen containing pyrane ring (C) (Balasundram *et al.,* 2006) (Figure 2).

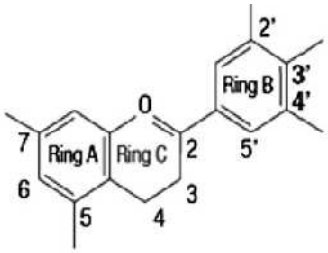


Figure 2. Basic monomeric structure of flavonoids (D'Archivio *et al.,* 2007).

Flavonoids are divided into anthocyanins and anthoxanthins. Anthocyanins are the molecules that posses some colour pigments such as red, blue and purple. Anthoxanthins are colourless or white to yellow molecules and are subdivided into five groups. These are flavones, flavonols, flavanones (or catechins), isoflavononones and flavanols (flavan-3-ols), that result of variations in the substitution patterns of ring C (Hollman and Katan, 1999). Substitutions to rings A and B give rise to different compounds within each class of flavonoids (Pietta, 2000). These substitutions may include oxygenation, alkylation, glycosylation, acylation, and sulphonation (Balasundram *et al.,* 2006).

The chemical structures of the main classes of flavonoids are presented in figure 3.

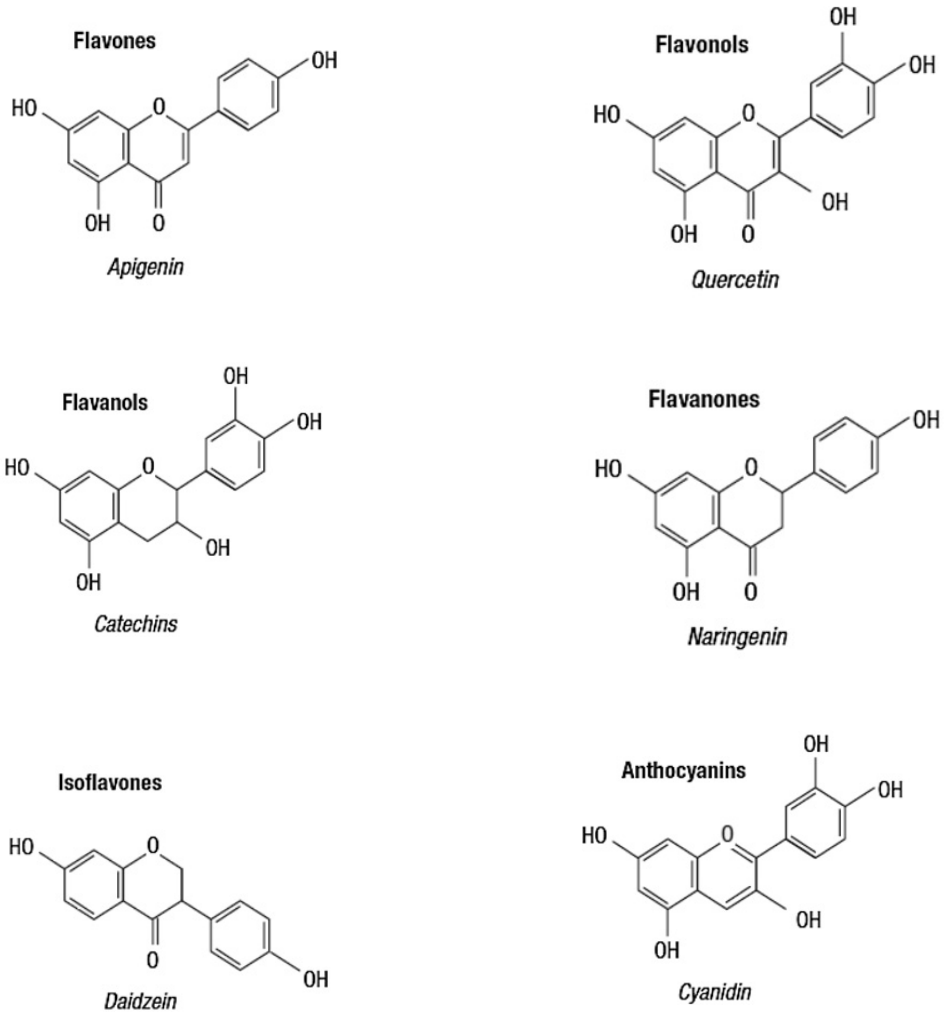


Figure 3. Chemical structures of flavonoids (D'Archivio *et al.,* 2007).

- **Flavones** are phenolic compounds that contain one carbonyl group (Cowan, 2003). A variety of substitutions is possible providing a wide array of natural and synthetic compounds; these include hydroxylation, methylation, 0- and C-alkylation and 0 and C-glycosylation. Most flavones occur as 7-0-glycosides (Balasundram, 2006). Flavone' distribution appears to be limited to only a few plant families, with the main flavones in the diet being apigenin and luteolin. They have been identified in celery (apigenin), sweet red pepper (luteolin), parsley and other herbs (Hertog *et al*., 1992).
- **Flavonols** are the most common type of flavonoids. The addition of 3-hydroxyl group to the flavones yields a flavonol (Cowan, 2003). Quercetin, kaempferol and myricetin are three widely distributed flavonols. Onion, apple, wine and tea are examples of foods which are high in their flavonol levels (King and Young 1999).
- **Flavanols (flavan-3-ol)** are flavonoids lacking the 2,3-double bond and the 4-one structure (Vermerris and Nicholson, 2006). Flavan-3-ols range from the simple monomers (+)-catechin and its isomer (-)-epicatechin, to the oligomeric and polymeric proanthocyanidins, also known as condensed tannins. In addition to forming complexes with other flavan-3-ols, they are hydroxylated to form the gallocatechins, and also undergo esterification with gallic acid. Furthermore, methylation, prenylation and O-glycosylation reactions have all been reported (Porter, 1992). (+)-Catechin and (-)-epicatechin are found in various fruits and vegetables such as apples, pears, grapes and peaches (Arts et al., 2000a), with the highest concentrations of catechins found in tea and red wine (Arts *et al.,* 2000b, Bell *et al.,* 2000).
- **Flavanones** are characterised by the presence of a saturated three-carbon chain and an oxygen atom in the 4 position of the C ring. They are generally glycosylated by a disaccharide in the 7 position of the A ring. Flavanones are present in high concentrations only in citrus fruit, but they are also found in tomatoes and certain aromatic plants such as mint. The main aglycones are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons (Peterson *et al*., 2006).

- **Isoflavones** have the B ring located in the 3 position of the C ring (Cowan, 2003). Isoflavones are the phenolic constituents that are mostly specific for legume family, especially for soybeans. The most famous isoflavones are daidzein and genistein (King and Young, 1999). These compounds have received much attention due to their putative role in the prevention of breast cancer and osteoporosis (Tapiero *et al.,* 2002).
- **Anthocyanins** are water-soluble vacuolar pigments that may appear as red, purple, or blue depending on pH. When anthocyanidins are found in their glycoside form (bonded to a sugar moiety) they are known as anthocyanins. Anthocyanidins are the basic structures of anthocyanins. Anthocyanidins (or aglycons) consist of an aromatic ring A bonded to an heterocyclic ring C that contains oxygen, which is also bonded by a carbon–carbon bond to a third aromatic ring B (Konczak and Zhang, 2004). Anthocyanins are responsible for the red, blue or violet colour of edible fruits including grapes, plums and berries, with levels increasing during fruit maturation (Dey and Harborne, 1993). The most common anthocyanidins include pelargonidin, cyanidin, delphinidin and malvidin (Bureau *et al.*, 2009).

2.3.1.2. Phenolic acids

Phenolic acids constitute about one-third of the dietary phenols, which may be present in plants in free and bound forms (Rice-Evans *et al*., 1996; Robbins, 2003). Bound-phenolics may be linked to various plant components through ester, ether, or acetal bonds (Zadernowski *et al*., 2009). Phenolic acids can be categorized in two subgroups, hydroxybenzoic and hydroxycinnamic acids (Figure 4).

- **Hydroxybenzoic acids** have in common the C_6–C_1 structure and include gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids. Gallic acid is the major hydroxybenzoate and is synthesised from phenylalanine via 3-dehydroshikimic acid. It is converted to ellagic acid and a range of gallotannins, with the formation of hydrolysable tannins (polymers of gallic and ellagic acids). Black tea and red wine

provide rich dietary sources of gallic acid (Dunfresne and Fransworth, 2001; Soleas *et al.* 1997).

- **Hydroxycinnamic acids** are aromatic compounds with a three-carbon side chain (C6–C3), caffeic, ferulic, p-coumaric, and sinapic acids being the most common representatives (Bravo, 1998). Caffeic acid occurs in foods mainly as an ester with quinic acid or chlorogenic acid (5-caffeoylquinic acid). Coffee is a major dietary source of chlorogenic acid in the diet with dietary intakes estimated at 0.5 -1 g/day (Clifford, 2000).

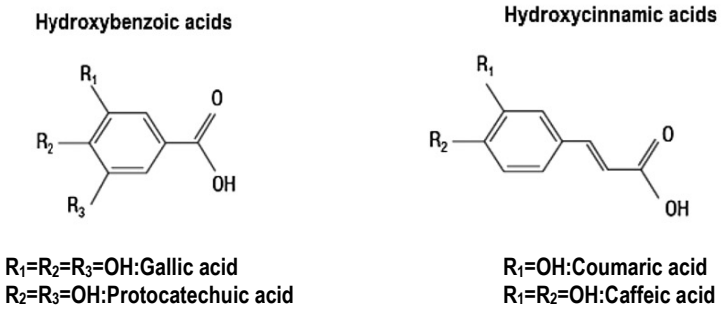


Figure 4 Chemical structures of phenolic acids (D'Archivio *et al.,* 2007)..

2.3.1.3. Tannins

Tannins are relatively high molecular weight compounds (molecular weight between 500 and 3000 Da), which constitute the third important group of phenolics. They may be subdivided into hydrolysable ((polyesters of gallic acid and polysaccharides) and condensed tannins ((polymerized products of flavan-3-ols and flavan-3,4-diols, or a mixture of the two) (Porter, 1992). Other type of tannins may also be seen as a combination of these two basic structures (Liao, *et al*. 2003).

- **Hydrolyzable Tannins** contain a central core of polyhydric alcohol such as glucose and hydroxyl groups (Figure 5) which are esterified either partially or wholly by gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins). After hydrolysis by acids, bases or certain enzymes, gallotannins yield glucose and gallic acids. The

hexahydroxydiphenic acid of ellagitannins undergoes lactonization to produce ellagic acid (Chung, *et al.* 1998).

- **Condensed Tannins** are structurally more complex than hydrolyzable tannins. Their complete structures are yet to be determined. They are mainly the polymerized products of flavan-3-ols and flavan-3,4-diols or a mixture of the two. The polymers, referred to as "flavolans", are popularly called condensed tannins. Condensed tannins are widely distributed in fruits, vegetables, forage, plants, cocoa, red wine and certain food grains such as sorghum, finger millets, and legume (Chung*, et al.* 1998).

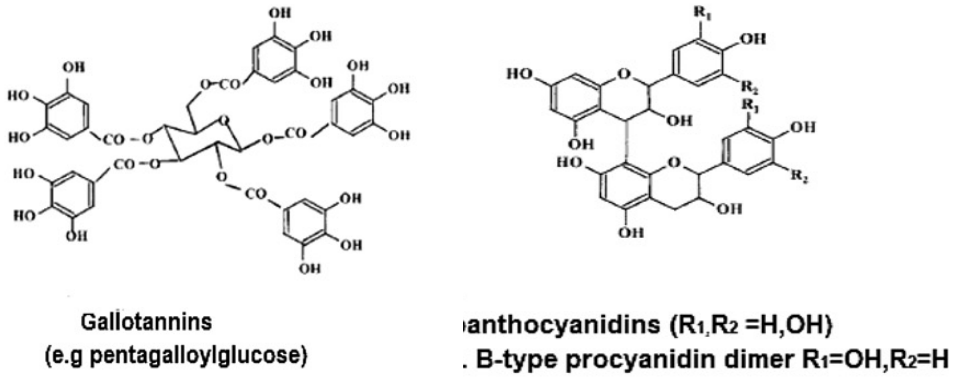


Figure 5. Chemical structures of tannins (D'Archivio *et al.,* 2007).

2.3.1.4. Stilbenes and lignans

- **Stilbenes** have a C_6-C_2-C_6 structure and are known to be phytoalexins, a class of antibiotic compounds produced as a part of a plant's defence system against disease (Bavaresco, 2003, Delmas *et al.,* 2006). Low quantities of stilbenes are present in the human diet, and the main representative is resveratrol (Figure 6), that exists in both cis and trans isomeric forms, mostly in glycosylated forms (Delmas *et al.,* 2006) Grapes, peanuts and their products are considered the most important dietary sources of resveratrol, with levels of 0.02-1.79 µg g^{-1} and 0.6-8 µg mL^{-1} reported in peanuts and red wine (Sanders *et al.,* 2000*;* Cornwell *et al*., 2004; Vingtdeux *et al*., 2008).

- **Lignans** are produced by oxidative dimerisation of two phenylpropane units; they are mostly present in nature in the free form, while their glycoside derivatives are only a minor form. The interest in lignans and their synthetic derivatives is growing because of potential applications in cancer chemotherapy and various other pharmacological effects (Saleem *et al*., 2005).

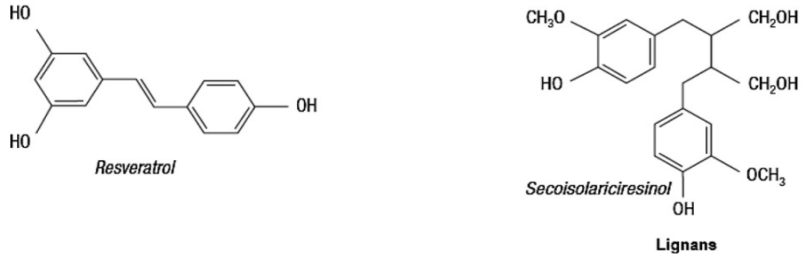


Figure 6. Chemical structures of stilbenes and lignans (D'Archivio *et al.,* 2007).

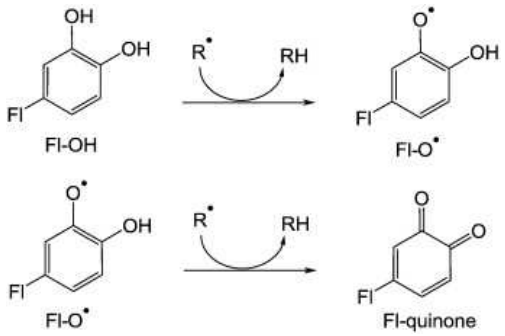
2.3.2. Antioxidant activity

Although the body has a number of endogenous defences, antioxidants from dietary sources also have an important role to play in quenching free radicals and in preventing damage. In addition to the common dietary antioxidants (vitamins C and E), phenolic compounds are purported to act as potent antioxidants in biological systems in vitro. The antioxidant capacities of many flavonoids are much stronger than those of vitamins C and E (Prior and Cao, 2000), e.g. proanthocyanidins showed antioxidant power 20 times greater than vitamin E and 50 times greater than vitamin C (Shi *et al*., 2003).

The protective effects have been attributed to their capacity to transfer electrons to free radicals, to chelate metal catalysts (Ferrali *et al.,* 1997; Pietta, 2000; Perron and Brumaghim, 2009), to activate antioxidant enzymes (Elliot *et al*., 1992; Nijveldt *et al.* 2001), to inhibit oxidases (Cos *et al.,* 1998; Heim *et al*., 2002), to reduce α-tocopheryl radicals (Hirano *et al.,* 2001; Heim *et al*., 2002; Zhou *et al*., 2005; Santos-Buelga *et al.* 2010), to mitigate oxidative stress caused by nitric

oxide (Acker *et al*., 1995), to increase uric acid levels (Lotito and Frei, 2006) and to increase antioxidant properties of low molecular antioxidants (Yeh *et al*., 2005).

Polyphenols have been found to be strong primary antioxidants, acting by chain-breaking mechanism, which can neutralise free radicals by donating hydrogen atoms or electrons to free radicals resulting in the formation of more stable products, as referred above. When these antioxidants, e.g. flavonoids (Fl-OH), are present in trace amounts, they can delay or inhibit the initiation step by reacting with a radical (eq. 1) and, as a consequence, reduce the radical chemical species (R•) to a non-radical products (RH), being themselves converted into oxidised radical (Fl-O •) (Procházková *et al*., 2011).



The formed radical should have low reactivity and, thus, be unable to react with biological components. However, free radicals formed from the antioxidant (Fl-O•) can be connected to other more reactive radicals (R•), acquiring a stable quinone structure, i.e. a stable non radical complex, Fl-quinone, (eq. 2), thus stopping the chain reactions. More frequently, they act as direct radical scavengers of the lipid peroxidation chain reactions (chain breakers) (Rice-Evans *et al.,* 1996; Pietta, 2000; Guo *et al.,* 2009).

Together with scavenging free radicals, polyphenols are also known as metal chelators. Polyphenols can act by a secondary mechanism (preventative), by slowing the oxidation rate of the initiation step (Antolovich, *et al*., 2002), as referred above. Chelation of transition metals such as Fe^{2+} can directly reduce or inhibit the formations of free radicals through the Fenton reaction, thereby avoiding its catalyzing effect on the formation of ROS, thus preventing

oxidation caused by highly reactive hydroxyl radicals (Ferrali *et al*., 1997; Pietta, 2000; Halliwell and Gutteridge, 2007; Perron and Brumaghim, 2009).

It has been found that polyphenols can actually function as a co-antioxidant (synergist), and are involved in the regeneration of essential vitamins. As an example, caffeic acid and p-coumaric acid can regenerate vitamin E by reducing α – tocopheryl radicals, which are formed when the vitamin E yield hydrogen atoms to the lipid oxidation chain reaction (Hirano *et al*., 2001; Heim *et al.*, 2002; Zhou *et al*., 2005; Santos-Buelga *et al*. 2010).

The antioxidant activities of polyphenols are related to their structure, and a number of important structural determinants have been identified. In the case of phenolic acids, the antioxidant activity depends on the numbers and positions of the hydroxyl groups in relation to the carboxyl functional group (Rice-Evans *et al*., 1996; Robards *et al*., 1999). Monohydroxybenzoic acids with the –OH moiety at the ortho- or para-position to the –COOH show no antioxidant activity, though the same is not true for the m-hydroxybenzoic acid (Rice-Evans *et al*., 1996). The antioxidant activity of phenolic acids increase with increasing degree of hydroxylation, as is the case of the trihydroxylated gallic acid, which shows a high antioxidant activity. However, substitution of the hydroxyl groups at the 3- and 5-position with methoxyl groups as in syringic acid reduces the activity (Rice- Evans *et al*., 1996). Hydroxycinnamic acids exhibit higher antioxidant activity compared to the corresponding hydroxybenzoic acids (Andreasen *et al*., 2001). The higher activity of the hydroxycinnamic acid could be due to the CH=CH–COOH group, which ensures greater H- donating ability and radical stabilisation than the –COOH group in the hydroxybenzoic acids (Rice-Evans *et al.,* 1996).

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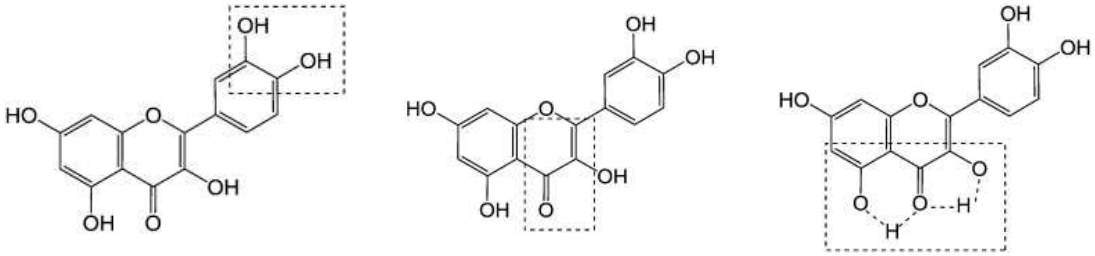
The antioxidant activity could be increased by polymerization of flavonoid monomers, e.g. proanthocyanidins (also known as condensed tannins), the polymers of catechins, are excellent antioxidants due to the high number of hydroxyl groups in their molecules. The glycosylation of flavonoids reduces their antioxidant activity when compared to the corresponding aglycons (Rice-Evans *et al*., 1996; Mishra *et al*., 2003).

Bors et al., 1990 proposed the following structural determinants for effective radical scavenging properties of flavonoids:

- The ortho-dihydroxy (catechol) structure in the B-ring, for electron delocalization which confers greater stability to aroxyl radicals. This is an obvious radical target site for all flavonoids with a saturated 2,3-double bond (Figure 7a);
- The 2,3-double bond in conjugation with a 4-oxo function in the C-ring , which is responsible for electron delocalisation from the B-ring, enhances the radical scavenging capacity of flavonoids (Figure 7b);
- The additional presence of both 3 and 5 hydroxyl groups provide hydrogen binding to the oxo group, for maximal radical scavenging capacity and strongest radical absorption (Figure 7c).

Moreover, the ability of flavonoids to donate electrons and stop chain reactions is attributed to the phenolic hydroxyls with activity increasing with the number of -OH groups in the A and B rings, particularly the 3', 4'-OH of the ring B and the meta arrangement of -OH groups at the 5 and 7 positions of ring A are reported to further increase the antioxidant activity of a compound (Frankel, 1999).



(a) **(b)** **(c)**

Figure 7. **(a)** An ortho-dihydroxy (catechol) structure in the B ring; **(b)** 2,3-Double bond in conjugation with a 4-oxo function in the C ring; **(c)** Hydroxyl groups at positions 3 and 5 (Procházková *et al*., 2011).

2.4. Wine and tea as natural source of phenolic antioxidant compounds

As widely accepted by the scientific community, beverages such as fruit juices, coffee, tea and wines are important sources of dietary phenolic antioxidants (Makris *et al.,* 2007; Leopoldini *et al.*, 2011). Table 3 provides some information about the total polyphenols content (TPC) of some beverages.

Table 3. Total polyphenols content of different beverages.

Beverage type	Total phenolics content	References
Red wines		
Portuguese	1724-1939[a]	Paixão *et al*., 2007
Spanish	1869[a]	Sanchez-Moreno *et al*., 2002
French	1847-2600[a]	Landrault *et al.,* 2001
Italian	4177-3314[a]	Minussi *et al.,* 2003
Rosé wines		
Portuguese	665[a]	Paixão *et al*., 2007
Spanish	486.4-329.6[a]	Sanchez-Moreno *et al.,* 2002
French	673-482[a]	Landrault *et al*., 2001
Italian	1304[a]	Minussi *et al.,* 2003
White wines		
Portuguese	282-434[a]	Paixão *et al*., 2007
Spanish	139.2-292.7[a]	Sanchez-Moreno *et al*., 2002)
French	262-1425[a]	Landrault *et al.,* 2001)
Italian	439-610[a]	Minussi *et al*., 2003)
Tea		
Green tea	65.8-106.2[b]	Khokhar and Magnusdottir, 2002
Black tea	80.5-134.9[b]	Khokhar and Magnusdottir, 2002
Coffee		
Instant coffee	146-151[b]	Lakenbrink *et al*.,2000
Ground coffee	52.5-57[b]	Lakenbrink *et al*.,2000
Commercial juices		
Grapefruit	535[a]	Gardner *et al.,* 2000
Orange	755[a]	Gardner *et al*., 2000
Apple	339[a]	Gardner *et al*., 2000
Pineapple	358[a]	Gardner *et al*., 2000

[a] mg gallic acid quivalents L^{-1}.

[b] mg gallic acid equivalents g^{-1} dry matter.

Wine is one of the world's most popular alcoholic beverages that contain a large amount of different polyphenols extracted from grapes *(Vitis vinifera*) (common grape vine), during the processes of vinification. The polyphenolic composition of wines is more complex as compared to their corresponding grape berries, because of the numerous reactions involving phenolic compounds that occur during the wine making and maturation processes (enzymatic and chemical oxidation reactions, condensation reactions, hydrolysis, etc.) (Monagas *et al*., 2005). The polyphenolic content of red wines differs essentially from that of white wines due to differences in the composition of red and white grapes, and also due to those in the vinification technology used (Bravo, 1998; Alén-Ruiz *et al.,* 2009). Table 4 showing the concentration of major phenolic compounds in red and white wines from California (USA) (Frankel *et al.,* 1995).

Phenolic compounds in wine are grouped within the following major classes: flavanols ((+) catechin, (-)-epicathechin, etc.), flavonols (quercetin, rutin, myricetin, etc.), anthocyanins (the most abundant is malvidin-3-O-glucoside), oligomeric and polymeric proanthocyanidins (condensed tannins), phenolic acids (gallic acid, caffeic acid, p-coumaric acid, etc.), stilbenes (trans-resveratrol), although it is important to keep in mind that wine contains many others polyphenols (Arts *et al.,* 2000b; Bell *et al.,* 2000; Monagas *et al.,* 2005). Resveratrol is the most famous polyphenolic compound occurring in grapes and wine. The levels of resveratrol found in wine vary greatly, but is generally more abundant in red wines (Sanders *et al*., 2001; Cornwell *et al*., 2004; Vingtdeux *et al.,* 2008).

Table 4. Concentration of major phenolic compounds in red and white wines (Frankel *et al.,* 1995).

Category	Phenolic compound	Red wine (mg L^{-1})[a]	White wine (mg L^{-1})[a]
Flavanols	(+)-catechin	191	35
	(-)-epicathechin	82	21
Hydroxybenzoic acids	Gallic	95	7
Anthocyanins	malvidin-3-O-glucoside	24	1
	cyanidin	3	0
Flavonols	rutin	9	0
	quercetin	8	0
	myricetin	9	0
Hydroxycinnamic acids	caffeic	7.1	2.8
Stilbenes	resveratrol	1.5	0
Average levels of total phenolic content[a]		2567	239

[a] mg gallic acid equivalents L^{-1}.

Phenolic compounds are major wine constituents that are responsible for some of the major organoleptic properties of wines, in particular colour, astringency, acerbity, flavour, and high antioxidant capacities (Minussi *et al.,* 2003; Rastija *et al*., 2009; Xia *et al.*, 2010). The antioxidant activity of phenolic compounds is directly related to their chemical structure, which can stabilize free radicals, as referred above. Those that partake in cellular degenerative processes can cause atherosclerosis, cancer and other diseases. Red wine may be more effective in combating these diseases, probably due to higher number of phenolic compounds such as catechin and gallic acid. The phenolic compounds of wine, and particularly the flavanols (i.e. catechins), have been the centre of attention in recent studies since their relation to the beneficial effects of a moderate consumption of wine was observed; often known as the “French Paradox” which refers to the hypothesis that red wine protects the French from the adverse cardiovascular effects of a fatty diet (Bleich *et al.,* 2001; Cornwell *et al*., 2004; Rodrigo *et al*., 2011). Phenolic compounds present in red wine cause an increase in serum total antioxidant capacity when ingested which thereby inhibit low-density lipoprotein (Whitehead and Robinson, 1995; Gorelik *et al*., 2005; Gorelik *et al.,* 2008; Boban and Modun, 2010), and reduce the risk of

cardiovascular disease. The antioxidative properties may also exert a chemopreventive role toward degenerative diseases, as well as act as preventative agents against skin cancer and other diseases (Stockley, 2009).

Tea derives from the leaves of the plant *Camellia sinensis* and is one of the most popular beverages consumed worldwide. The green tea plant is commonly grown in India, China, Japan, Taiwan, Sri Lanka, Indonesia and Central Africa, where it is produced and then exported to the rest of the world. The polyphenol composition of tea varies depending on the species, the season, the age of the plant, the conditions of cultivation, the manufacturing process and the storage methods (Prior and Cao, 1999). Three different types of tea: green (no “oxidation”), oolong (moderate “oxidation”), and black (complete “oxidation”) tea can be derived from this plant (Vinson *et al*., 2004).

Phenolic compounds are major tea constituents (Yao *et al.,* 2004). Tea is characterised by the presence of the polyphenolic catechins including: epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin. Flavonols, including quercetin, kaempferol, myricitin and their glycosides, as well as phenolic acids, including mainly caffeic, gallic, and quinic acids are also present in tea (Häkkinen *et al.*1999; Dalluge and Nelson, 2000; Gramza and Korczak, 2005; Khan and Mukhtar, 2007; Heiss and Heiss, 2007). The tea catechins are the major constituents of fresh tea leaves (30–42% of the dry weight), being oxidised during fermentation to yield a complex mixture of secondary polyphenols including theaflavins, theasinensins and oolongtheanins (Hashimoto *et al.,* 2003).

Research findings suggest that the polyphenolic compounds, EGCG found primarily in green tea, and theaflavin-3,3′-digallate, a major component of black tea, are the two most effective anti-cancer factors found in tea (Nanjo *et al*., 1999). The potential health benefits associated with tea consumption have been partially attributed to the antioxidative property of tea polyphenols (Mukhtar and Ahmad, 2000). The radical quenching ability of green tea is usually

higher than that of black tea and has been justified by high catechin levels (4-6 times) in green tea than in black tea (Pham-Huy et al., 2008). Among tea catechins, EGCG is most effective in reacting with most ROS. A significant rise in plasma antioxidant capacity was detected after brewed green tea or black tea solids were consumed (Leenen *et al*., 2000). It has been reported that green tea consumed within a balanced controlled diet improve the overall antioxidative status and protect against oxidative damage in humans (Erba *et al.*, 2005). However, Sumpio *et al.* (2006) concluded that green tea consumption may be the explanation of the "Asian paradox." *i.e*., green tea may protect Asians from the adverse effects of smoking. Many people in Asia are smokers, yet there is a lower incidence of cardiovascular disease and cancer than in many countries in which fewer people smoke. The authors noted that, the average 1.2 litres of green tea consumed each day by many Asians provides the antioxidant EGCG that may prevent LDL oxidation, which has been shown to play a key role in the pathophysiology of arteriosclerosis. EGCG also reduces the amount of platelet aggregation, regulates lipids, and promotes proliferation and migration of smooth muscle cells, which are all factors in reducing cardiovascular and cancer diseases. Tea preparations have been shown to trap ROS, such as superoxide radical, singlet oxygen, hydroxyl radical, peroxyl radical, nitric oxide, nitrogen dioxide and peroxynitrite, reducing their damage to lipid membranes, proteins and nucleic acids in cell-free systems. Green and black tea can inhibit the oxidation of lipoproteins induced by Cu_2^+ *in vitro* (Leenen *et al*., 2000; Erba *et al.*, 2005; Pham-Huy et al., 2008).

2.5. Methods for identification and quantification of phenolic compounds and *In vitro* evaluation of antioxidant activity in wine and tea

This review examines more extensively the analytical methods/assays that were used in the experimental work for the analysis of phenolic compounds, as well as the radical scavenging activity in wine and tea.

2. 5.1. Analysis of phenolic compounds

2.5.1.1. Spectrophotometric determination of total phenolic content by Folin-Ciocalteu colorimetry assay

The Folin–Ciocalteu (FC) assay is one of the oldest methods designed to determine the total content of phenolic compounds, which are responsible for the total antioxidant capacity of a specific sample. This assay was originally intended to analyse protein by taking advantage of the presence of the phenolic group in tyrosine. Later, Singleton adapted this method to wine analysis (Singleton and Rossi, 1965) and wrote two major reviews on its use (Singleton, 1974; Singleton *et al.,* 1999). The FC procedure is one of the standard procedures in wine analysis (OIV, 1990), and also is widely used to estimate the phenolic content in tea samples (Wiseman *et al*., 2001; Samaniego-Sánchez *et al*., 2011; Jayasekera *et al*., 2011).

The colorimetric assay is based on a chemical reduction of the Folin-Ciocalteu reagent (FCR), composed of a mixture of phosphotungstic and phosphomolybdic acids, which are reduced by the oxidation of the phenols, forming a mixture of blue oxides of tungsten and molybdenum. The blue coloration exhibits a broad light absorption with a maximum at 765 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenolic compounds present in the sample. The method consists on a calibration with a pure phenolic compound, extraction of phenols from the sample, and the measurement of absorbance after the color reaction. Gallic acid is usually used as standard to produce the calibration curve. The total phenolic content is expressed in gallic acid equivalents (GAE) using units of mg L^{-1}. For any standard, the results must always be reported on an equivalent basis to avoid the perception that one is measuring the amount of the standard substance (Wrolstad *et al*., 2005).

The major disadvantage of FC assay is its low specificity due to the fact that the colour formation of FC reaction is based on FCR' chemical reduction, being this reaction general enough to allow the interference of a number of sources*, i.e*., the FCR does not only react with phenolic compounds, but also with any reducing substance, being that the colour reaction can occur with any oxidisable phenolic hydroxy group as many non-phenolic compounds (Singleton and Rossi, 1965; Beer et al., 2004; Wrolstad *et al.,* 2005). In wine, the principal interfering compounds are sulfur dioxide and ascorbate, though high levels of sugar indirectly enhance the readings of other analytes (Wrolstad *et al.,* 2005). For these reasons several authors consider this method not suitable for the determination of the "total phenolic content", unless interfering species, as sugars, are considered or removed. However, FC assay may be useful for screening purposes, because it consists of a simple reproducible technique, and for these reasons has been widely used when studying phenolic antioxidants.

2.5.1.2. Determination of phenolic profile by High performance liquid chromatography (HPLC-DAD)

High-performance liquid chromatography (HPLC) is an efficient tool used for the separation, identification, and quantification of individual phenolic compounds from complex mixtures, based on the compounds' variation in affinity for a resin packed in a column (Paixão *et al*., 2008; Ignat *et al*., 2011).

The chromatographic conditions of the HPLC methods include the use of, almost exclusively, a reversed-phase (RP) C18 columns, UV–Vis diode array detector, and a binary solvent systems consisting of a solvent A usually acidified water and a polar organic solvent B, such as acetonitrile or methanol (Zhang *et al.,* 2005, Pereira *et al*., 2010). RP-HPLC has become a dominating analytical tool for the separation and determination of polyphenols with different detection systems, such as diode array detector (DAD), mass or tandem mass spectrometry (Paixão *et al.,* 2008). The most frequent wavelengths used were 290 and 340 nm. Due to the fact, that some phenolic compounds show several absorption maxima, the use of simultaneous

multiple UV wavelengths (photodiode array) is recommended for identification purposes, and also because this detector offers chromatograms at any wavelength accompanied by the absorption spectrum of each eluted band. In this way, the absorption spectrum can be combined with retention parameters for the possible identification of an unknown compound as well as to measure the purity of the elution band in question (Ignat *et al.,* 2011). However, due to the disadvantages in detection limit and sensitivity, HPLC methods present limitations especially in complex matrix. Thus, an initial pre-concentration and purification of the polyphenols from complex matrix is crucial before the instrumental analysis by HPLC. Lower molecular mass polyphenols can be analysed by HPLC on reversed-phase or normal phase columns. However, these techniques are time and reagent consuming and can have poor resolution as the polymer chain length and structural diversity increase (Ignat *et al*., 2011).

HPLC technique identify individual polyphenols that exist in wine' solution in free form (Tarola *et al.,* 2007, Paixão *et al.,* 2008; Pereira *et al.,* 2010), bonded to sugars (Fang *et al*, 2007) or oligomers (proanthocyanidins, from dimers to hexamers) (Cosme *et al*. 2008; Vergara *et al.*, 2010), but they are not able to detect polyphenols that are highly polymerized or associated with macromolecules (Fulcrand *et al*., 2008).

HPLC technique was developed to analyse black and green tea constituents in the 1970s (Hefler and Cognan, 1976) and has shown effective separation, identification and quantification of the tea phenolic compounds (Wang *et al*., 2008; Horžić *et al*., 2009).

2.5.2. Radical scavenging activity

Numerous in vitro studies have been conducted to evaluate the total antioxidant activity (TAA) of wine and teas (Villaño *et al*., 2006; Staŝko *et al*.,2008; Mudnic *et al*., 2010; Piljac-žegarac *et al.*, 2010; Komes *et al*., 2010; Jayasekera *et al*., 2011; Andlauer and Héritier, Alarcón *et al*., 2008; Samaniego-Sánchez *et al.,* 2011). Each method is related to the generation of a different radical, acting through a variety of mechanisms and the measure of a range of end points at a

fixed time point or over a range. Two types of approach have been taken, specifically, the inhibition assays in which the extent of the scavenging of a pre-formed free radical by hydrogen or electron donation is the marker of the antioxidant activity, as well as assays involving the presence of antioxidant system during the generation of the radical (Re *et al*., 1999).

Basically, the methods for measuring TAA are generally classified into two categories, depending on the reaction mechanism (Huang *et al*., 2005):

- electron transfer (ET) assays, and;
- hydrogen atom transfer (HAT) assays.

ET based assays measure the reducing ability of the substrate (antioxidant); they include the Trolox equivalent antioxidant capacity (TEAC) assay, the ferric ion reducing antioxidant power (FRAP) assay and the 2,2-diphenyl-1- picrylhydrazyl assay (DPPH). The Folin-Ciocalteu method is an ET based assay that gives reducing capacity, which has normally been expressed as phenolic content (Prior, 2005). These assays are characterised by their ability to undergo single electron transfer, [indicator (oxidant) + e^- (from antioxidant) → reduced indicator + oxidized antioxidant]. The indicator is an oxidant, which extracts an electron from the antioxidant, causing the indicator to change colour. The degree of colour change is correlated with the sample's antioxidant concentration.

HAT-based assays apply a competitive design, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo-compounds. HAT-based assays include the crocin bleaching assay, the total peroxyl radical-trapping antioxidant parameter (TRAP) assay, and the oxygen radical absorbance capacity (ORAC) assay.

Trolox equivalent antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC) assays are the most popularly used ET and HAT methods, respectively.

Studies of radical scavenging activity in wine and tea samples are described in Table 5, giving principle of measurement, quantification and recent applications.

Table 5. *In vitro* scavenging capacity assays against stable, non-biological radicals and evaluation of TAA in wine and tea.

Principle	Assay	Principle of Measurement	Quantification	Recent Applications
Electron transfer (ET)	TEAC	ABTS•+ radical cation is reduced by antioxidants, causing absorbance decrease at 734nm	Trolox equivalents (TEAC, mM)	Villaño *et al*.,2006; Staško *et al.,*2008; Mudnic *et al*., 2010; Piljac-žegarac *et al.*, 2010; Komes *et al.,* 2010; Samaniego-Sánchez et al., 2011.
	DPPH	DPPH• radical is reduced by antioxidants, causing absorbance decrease at 515nm	EC_{50}, RSE, Trolox equivalents (TEAC,mM), ascorbic acid equivalents (mg/L^{-1})	Villaño *et al*., 2006; Staško *et al.,*2008; Piljac-žegarac *et al*., 2010; Jayasekera *et al*., 2011; Andlauer and Héritier, 2011.
	FRAP	The ferric 2,4,6-tripyridyl-*s*-triazine complex is reduced by antioxidants, causing absorbance increase at 593nm	Ferrous ions equivalents, Trolox equivalents (TEAC, mM)	Mudnic et al., 2010; Piljac-žegarac *et al.,* 2010; Komes *et al*., 2010; Andlauer and Héritier, 2011.
Hydrogen atom transfer (HAT)	ORAC	Free radicals are produced by AAPH and the fluorescent indicator protein *β*-PE is subsequently oxidized. Fluorescence is measured with emission and excitation wavelengths of 565 and 540 nm	Trolox equivalents (TEAC, μM)	Villaño *et al*., 2006; Alarcón *et al*., 2008.

TEAC: Trolox equivalent antioxidant capacity; $ABTS^{\bullet+}$: 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation; $DPPH^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl radical; EC_{50}: efficient concentration; RSE: radical scavenging efficiency; FRAP: ferric reducing antioxidant power; ORAC: Oxygen radical absorbance capacity.

As seen in table 5, several methods for determining the TAA in wine and tea have been used by many authors. Along with the DPPH method, the ABTS assay is one of the most extensively used antioxidant assay for beverages samples (Rice-Evans *et al*., 1996; Re *et al*., 1999; Floegel *et al*., 2011).

2.5.2.1. ABTS or TEAC (trolox equivalent antioxidant activity) assay

The ABTS radical [2,2'- azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] scavenging method was developed by Rice- Evans and Miller in 1994 and was modified by Re et al. in 1999. This assay undertakes the assessment of the total radical scavenging capacity based on the ability of a compound to scavenge the stable ABTS radical cation ($ABTS^{\bullet+}$). $ABTS^{\bullet+}$ is generated by the oxidation of ABTS with potassium persulfate ($K_2S_2O_8$).

The $ABTS^{\bullet+}$ radical typically has a blue/green colour with absorption maxima at 414, 645, 734 and 815 nm (Re *et al.,* 1999). When there are antioxidant compounds in the reaction medium, free radicals will be captured by the pre-formed radical cation, which will be translated by a loss of colour and therefore a reduction in absorbance, corresponding quantitatively to the concentration of antioxidants present (Figure 8). The extent of decolourisation as percentage inhibition of the ABTS' radical cation is determined as a function of concentration and time, being calculated in relation to the reactivity of Trolox (a water soluble analogue of vitamin E) as a standard, under the same conditions. The activity is expressed in terms of the Trolox-equivalent antioxidant capacity of beverage samples (TEAC, mmol L^{-1}).

The ABTS assay is considered an easy and accurate method for measuring the antioxidant activity. However, this assay has been criticized, because ABTS is not a physiological radical source, and thus may not accurately represent *in vivo* effects. In spite of this, the excellent spectral characteristics, the solubility in both organic and aqueous media, and the stability in a wide pH range, makes this assay one of the most extensively used for the estimation of the antioxidant activity of pure compounds, both liophilic and water soluble (Nemadis *et al*, 2004).

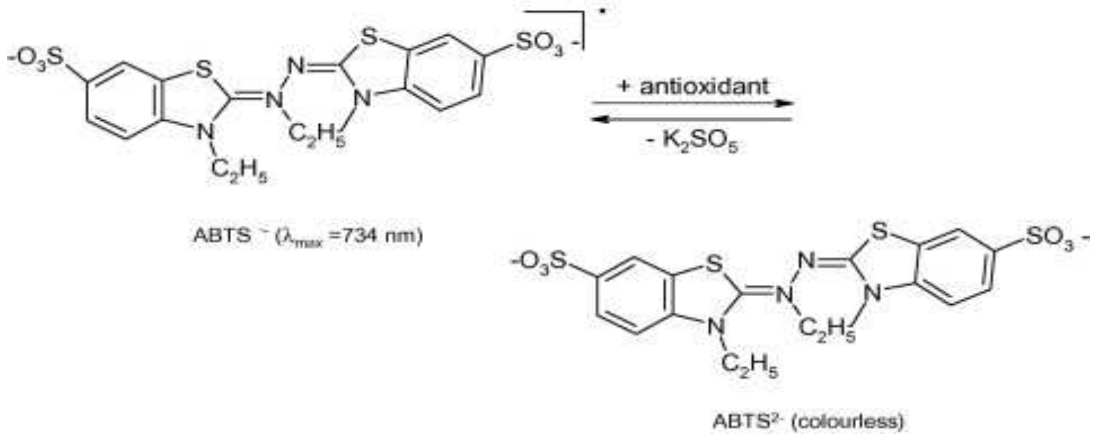


Figure 8. Reaction of the ABTS radical in the presence of the antioxidant compound during the ABTS assay (Zuleta *et al*., 2009).

2.5.3. Electrochemical techniques

The electrochemical techniques with controlled-potential (potentiostatic methods) have been applied in quantitative and qualitative analysis of polyphenolic compounds. Basically, the electrode potential drives redox reactions on species of interest and the resultant current is measured (Wang, 2001; Skoog *et al*., 2004). The current generated is proportional to the concentration of an electroactive species. Amongst the potentiostatic techniques are cyclic voltammetry and amperometry. These electrochemical techniques were performed in this work and will be presented briefly below.

2.5.3.1. Cyclic voltammetry method

Cyclic voltammetry (CV) is an effective and versatile electroanalytical technique which allows to investigate the mechanism of redox and transport properties of a system in solution. This is accomplished with a three electrode arrangement whereby the potential relative to some *reference* electrode is scanned at a *working* electrode while the resulting current flowing through a *counter* (or *auxiliary*) electrode is monitored in an unstirred solution (Wang, 2001).

In a typical cyclic voltammetry experiment, the potential of the working electrode in solution is linearly cycled from an initial potential to a final potential then back to the initial potential. The potential extrema are known as the switching potentials. These potentials are chosen to include the region where oxidation and/or reduction of the analyte takes place, the redox reaction of interest is therefore cycled. When the potential of the working electrode is more positive than that of a redox couple present in the solution, the corresponding species may be oxidised (i.e. electrons going from the solution to the electrode) and produce an anodic current. Similarly, on the return scan, as the working electrode potential becomes more negative than the reduction potential of a redox couple, reduction (*i.e*. electrons flowing away from the electrode) may occur to cause a cathodic current. By IUPAC (*International Union of Pure and Applied Chemistry)* convention, anodic currents are positive and cathodic currents negative.

The current generated is proportional to the concentration of an electroactive species. A diagram of current generated versus the potential (voltammogram) can be plotted and used to identify the redox potential of the electroactive components and whether the redox reactions are reversible or not. This technique facilitates the comprehension of the electrochemical behaviour of a molecule (compound) (Skoog *et al.,* 2004). A typical voltammogram is shown in Figure 9. The scan shown starts at a slightly negative potential, *(A)* up to some positive switching value, *(D)* at which the scan is reversed back to the starting potential. The current is first observed to peak at *Ep,a* (with value *Ip,a*) indicating that an oxidation is taking place and then drops due to depletion of the reducing species from the diffusion layer. During the return scan the processes are reversed (reduction is now occurring) and a peak current is observed at *Ep,c* (corresponding value, *Ip,c*).

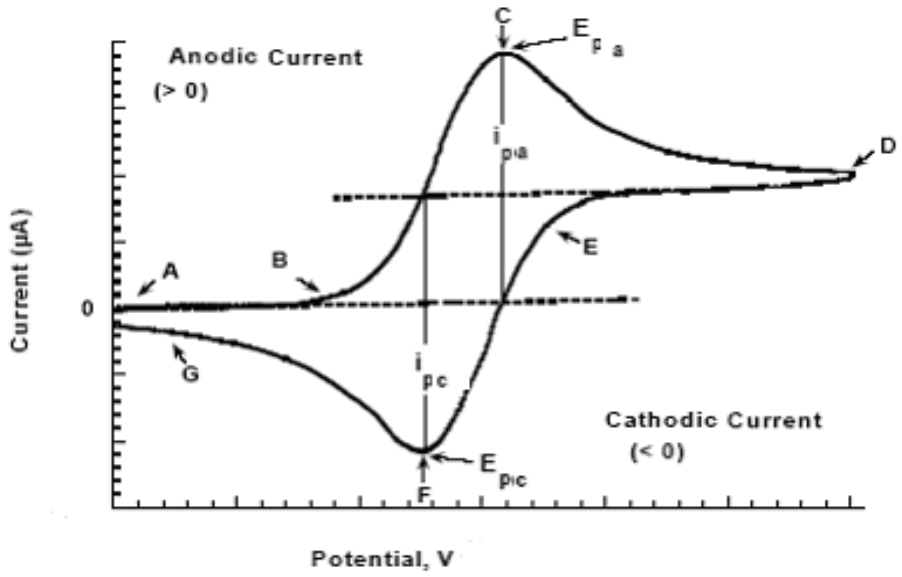


Figure 9. The expected response of a reversible redox couple during a single potential cycle (Wang, 2001).

The current is first observed to peak at $E_{p,a}$ (with value I_{pa}) indicating that an oxidation is taking place and then drops due to depletion of the reducing species from the diffusion layer. During the return scan the processes are reversed (reduction is now occurring) and a peak current is observed at $E_{p,c}$ (corresponding value, $I_{p,c}$). Providing that the charge transfer reaction is reversible, that there is no surface interaction between the electrode and the reagents, and that the redox products are stable, the ratio of the reverse and the forward current $I_{p,c}/I_{p,a}$ = 1.0. In addition, for such a system it can be shown that (Wang, 2001; Skoog *et al*., 2004):

1) the corresponding peak potentials $E_{p,a}$ and $E_{p,c}$ are independent of scan rate and concentration;

2) $(E_{p,a} + E_{p,c})/2$ is a good approximation of E_0', the formal potential for the redox reaction and;

3) $E_p = E_{p,a} - E_{p,c}$ should be close to 59/*n* mV (for a *n* electron transfer reaction) at all scan rates.

These parameters test the reversibility of a redox process and stability of the products; if any of these parameters is not satisfied, it is an indication that the system is not fully reversible.

Cyclic voltammetry (CV) is an instrumental tool that has been extensively applied for characterisation of polyphenolic compounds and determination of total polyphenolics in wine (Firuzi *et al.*, 2005; Makhotkina and Kilmartin, 2010), and also in tea (Kilmartin and Hsu, 2003; Piljac-Žegarac *et al*., 2010).

Electrochemical methods for antioxidant assays have emerged recently and are based on the fact that one of the modes of action of antioxidants is the ability to donate hydrogen atoms to free radicals to neutralise them, eq. 1,

$$AH \rightarrow A^{\bullet} + H^{\bullet} \quad (1)$$

On the other hand, being phenolic compounds electroanalytically active compounds, they are easily oxidised at inert electrodes. Although the anodic potential ($E_{p,a}$) is a measure of oxidisability according to the eq. 2, electrochemical data provide useful information about the free radical scavenging activities of phenolic compounds because of the similarities of these two reactions. Thus, the oxidation potential of phenolic compounds determined by CV is considered a good measure of their antioxidant activities (Firuzi *et al.*, 2005; Makhotkina and Kilmartin, 2010).

$$AH \rightarrow A^{\bullet} + H^{+} + e^{-} \quad (2)$$

CV scans (voltammograms) may be used to determine the ability of phenolic compounds to donate an electron around the oxidation potential of the anodic peak. When applied to samples containing antioxidants, anodic peaks may be ascribed to individual compounds based on oxidation potential, $E_{p,a}$, while their concentrations are proportional to the obtained current intensity, $I_{p,a}$. A number of others parameters (e.g. the number of transferred electrons, the rate of the electrode reaction) can be extracted from the cyclic voltammograms to characterise phenolics as reducing agents. Thus, CV can be used for a rapid test of the antioxidant activity of phenolic compounds, and others electroanalytically active compounds (Chevion *et al*., 2000).

A commom problem encountered while performing cyclic voltammetry analysis is the adsorption of substances present in sample matrix on the electrode surface. Formation of these layers

leads to electrode fouling which modifies the voltammetric signal and suppresses the sensitivity of the electrode (Wang, 2001; Skoog *et al*., 2004).

2.5.3.2. *Amperometry*

In amperometry, the current is measured as a function of time, at a constant potential between a polarised working electrode and a reference electrode. The working electrode is the electrode where the the reaction of interest takes place. It is tipically made of an inert material, *e.g*. platinum, gold or carbon. Typical reference electrodes are silver/silver chloride (Ag/AgCl/KCl) and saturated calomel ($Hg/Hg_2Cl_2/KCl_{sat}$).The electrodes are usually in a stirred or flowing solution. The amperometric measurement technique is based on the electrochemical oxidation or reduction of certain chemical species, and involves the transfer of electrons between the chemical species and the electrode, thereby inducing a current flow. By selection of the appropriate applied potential, the particular species to be measured can often be selected. The limiting current, that is, the maximum current for the reaction at the applied potential selected for the amperometric measurement for the component of interest, is linearly related to the concentration of the electroactive species at fixed fluid velocity and temperature. In most cases of amperometric biosensor development all the required parameters were already obtained with this simple two electrode set-up. Otherwise, a three electrode set-up can be used, consisting of a working-, a reference- and a counter electrode (auxiliary electrode). In this case the counter electrode provides the current path in an electrochemical cell (Bard and Faulkner, 1980; Thévenot *et al*., 1999; Bagotsky, 2006).

Normally, this technique allows the detection of molecules or ions at concentrations as low as 10^{-9} M and has a dynamic range of 3 to 4 orders of magnitude (Turner *et al*., 1989; Thévenot *et al.,* 1999). Since in analytical measurements, very little material is consumed and, therefore, the bulk concentration of the electroactive species remains relatively constant, steady state conditions can be assumed (Bard and Faulkner, 1980). In case of biosensor response, the current is measured at various substrate concentrations and compared with the current when no

substrate is present. The difference between these two situations indicates biosensor activity (Turner *et al*., 1989).

Phenolic compounds can be determined electrochemically by means of a direct oxidation (chemical oxidation), as seen above, but this procedure is hampered by the need to apply a high overpotential (+0.8 to +0.95 *versus* Ag/AgCl) (Kochana *et al*., 2008).The high overpotential applied current causes a large background, and thus a high noise level. Furthermore, the direct oxidation of phenolic compounds can occur as a result of parallel reactions (*e.g.* quinone polymerisation) leading to formation of polymeric by-products that passivate the electrode surface.

The use of chemically modified electrodes (CMEs), such as biosensors, greatly minimizes this overpotential of reduction of electroactive species (product of the enzymatic oxidation) at the electrode surface at potentials around (0.0 to +0.2 *versus* Ag/AgCl) (Kochana *et al*., 2008). At this potential, the effect of interference caused by oxidation or reduction of other species present is minimized. This subject has been also addressed in sections 2.6.1 and 2.6.4.

2.5.4. Biosensor evaluation of the antioxidant activity

The determination of free radicals and antioxidants has been widely investigated in the food technology and human health fields. Traditional techniques such as spectrophotometry, or HPLC (Prior *et al*., 2005; Roginsky and Lissi, 2005) are expensive, reagent and time consuming. Thus, they are being replaced by other innovating technologies, such as biosensors, which are attractive and promising tools in polyphenols detection due to their unique characteristics such as specificity, sensitivity, low cost, miniaturization, easy automation, time saving, simplicity of operation and manufacturing (Gomes, *et al.* 2004; Fusco *et al*., 2010).

While the main objective of the medical field is one of evaluating the ability of some compounds to scavenge free radicals, food science research aims to detect and quantify them. In this

sense, two different types of biosensors are reported in the antioxidant assessment: the ones based on the “total phenol content” determination, and those based on the antioxidant capacity measurement, which is established on the determination of the free radical scavenging activity. The first type of biosensors measures the antioxidant activity, which corresponds to the rate constant of a single antioxidant against a given free radical, and the other type displays antioxidant capacity, which is the measurement of moles of a given free radical scavenged by a test solution, independently of the antioxidant present in the mixture (Mello and Kubota, 2007).
All biosensors developed for this purpose are electrochemical and use ROS in their configurations. ROS are not commercially available because of their highly reactive nature and their very short lifetime. Consequently, the first step in the development of such biosensors is their generation *in vitro* (Prieto-Simón *et al*., 2008).

Several amperometric biosensors for the detection of phenolic compounds (the main antioxidant compounds in food) have been developed on the basis of enzymes such as tyrosinase, laccase or peroxidase. These enzyme based biosensors allow the evaluation of the usually named “total phenol content” (Mello and Kubota, 2007; Prieto-Simón *et al*., 2008).

2.6. Laccase-based amperometric biosensor for determination of phenolic compounds

The main purpose of this review section is to provide information about the laccase-based amperometric biosensor used in experimental work. This polyphenolic biosensor was developed in our laboratory by Prof. Maria José Rebelo (Gomes and Rebelo, 2003; Gomes *et al*., 2004; Júnior and Rebelo, 2008), and many references about it can be found in the literature.

2.6.1. Amperometric biosensors

A biosensor can be defined as an analytical device that combines a biological material (tissues, cells, organelles or molecules (*e.g.* antibody, protein and enzymes) with an appropriate transducer (*e.g.* electrochemical, optic, calorimetric/thermometric, piezoelectric) capable of giving selective and/or quantitative analytical information (Turner *et al*., 1989; Terry *et al*., 2005). Schematical representation of a biosensor system is given in Figure 10.

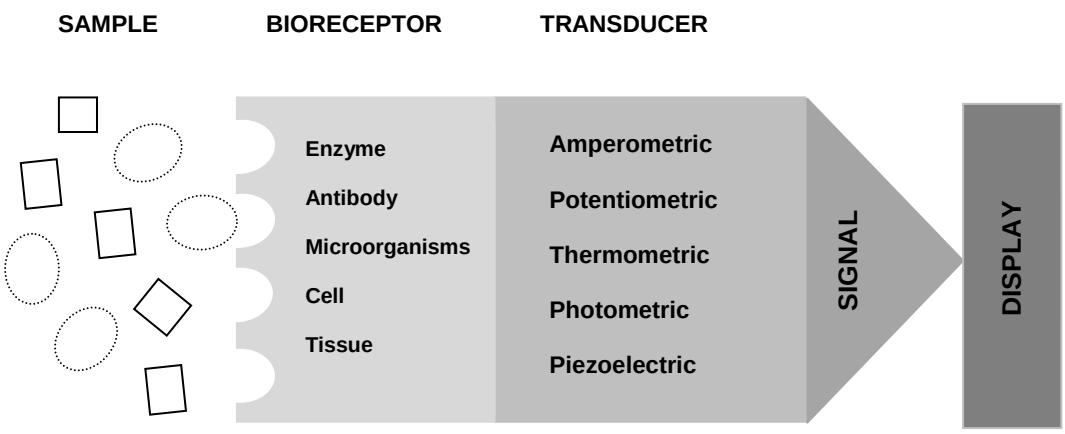


Figure 10. Biosensor system (*adapted from* Terry et al., 2005).

As it is shown on the above figure, biosensors are made up of three different but strictly connected elements, the biological recognition system (selector), the transducer and the detector (Thévenot *et al*., 1999).

- The biological recognition system (selector) translates information from the biochemical domain, usually an analyte concentration, into a chemical or physical output signal with a defined sensitivity. The main purpose of the recognition system is to provide the sensor with a high degree of selectivity for the analyte to be measured.
- The transducer transfers the signal from the output domain of the recognition system to a physically measurable signal.
- The detector permits to display the chemico-physical signal into a suitable form.

Biosensors are classified according to their biological recognition elements, or the mode of signal transduction or, alternatively, the combination of these two aspects (Thévenot *et al*., 1999).

The electrochemical biosensors are the most widely used type of biosensors and being based on the generation of an electrochemical signal during the interaction of the biocomponent with the analyte. According to the *International Union of Pure and Applied Chemistry* (IUPAC), an electrochemical biosensor is a “selfcontained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element” that is in direct contact with an electrochemical transducer (Thévenot *et al*., 1999).

In electrochemical biosensors, the transduction element is the electrode. These biosensors can be further divided into amperometric (current at constant potential, in redox reaction, as seen in section 2.5.3.2.), potentiometric (potential changes at constant current, (*e.g*. ion selective electrodes), and conductimetric biosensors (conductance changes in the ionic environment) according to the electrochemical property measured (Turner et al., 1989; Thévenot *et al*., 1999).

An amperometric biosensor may be more attractive due to its high sensitivity and wide linear range. It offers more precise and accurate results, and it is also not necessary to wait until the thermodynamic equilibrium is obtained and the response is a linear function of the concentration of the analyte. Therefore, amperometric enzymatic electrodes hold a leading position among the presently available biosensor systems. These devices combine the selectivity of the enzyme for the recognition of a given target analyte with the direct transduction of the rate of the biocatalytic reaction into a current signal, allowing a fast, simple and direct determination of various compounds. However, the selectivity of the amperometric devices is only governed by the redox potential of the electroactive species present. Consequently, the current measured by the instrument can include the contributions of several chemical species (Mello and Kubota, 2002).

In 1962, Leland Clark developed the biosensor' concept which lead to the creation the biosensor used for glucose' analysis employing glucose oxidase enzyme (GOD) with the Clark' oxygen electrode, which was based on the oxygen consumption monitoring. The formation of the product or consumption of reagent can be monitored to measure the analyte concentration. These biosensors are classified as the first generation. Amperometric biosensors modified with specific mediators are referred as the second generation biosensors. Mediators are redox substances that facilitate the electron transfer between the enzyme and electrode. The direct enzyme-electrode coupling or mediatorless biosensors based on direct electron transfer (DET) mechanisms are classified as the third generation. In this case, the electron is directly transferred from the electrode to enzyme and to the substrate molecule (or vice versa). In this mechanism the electron acts as a second substrate for the enzymatic reactions and result in the generation of a catalytic current. The substrate transformation (electrode process) is essentially a catalytic process (Shleev *et al*. 2004; Mello and Kubota, 2002).

Electroanalytical techniques are fairly sensitive and currents as low as 10^{-10} A can be recorded by commercial devices. However, the introduction of a layer incorporating the enzyme over the surface of the electrode decreases the sensitivity of the electrode by one or two orders of magnitude, due to the additional diffusion resistance. Therefore, for the measurement of analyte

concentrations in the nanomolar range an increase of sensitivity of the enzyme electrode is required. One way to solve this problem is the continuous regeneration of the analyte in cyclic reactions, as it is verified with 3rd generation biosensors.

2.6.1.1. Characteristics of the amperometric biosensor response

The following list contains parameters which can be used to characterize the performance of amperometric biosensors response, according to IUPAC. These criteria include calibration characteristics (sensitivity, linearity, detection limit, and response time), selectivity, reproducibility, stability and lifetime (Thévenot *et al*., 1999; Gründler, 2007).

- **Sensitivity:** change in the measurement signal per concentration unit of the analyte, i.e. the slope of a calibration graph.
- **Linearity:** range of concentration of the substrate over which the sensitivity of the electrode is constant with a specific variation.
- **Limit of detection (LOD):** the lowest concentration value which can be detected by the amperometric biosensor, under specified conditions, *i. e*. the smallest measure of the intensity of the current that can be detected with reasonable certainty. This quantity is then related to a multiple k of the standard deviation of the blank ``according to the confidence level desired'', with the general recommendation of k=3.
- **Response time:** the time for an amperometric electrode to respond from zero concentration to a step change in concentration. Usually specified as the time to rise to a definite percentage of the final value. Thus, *e.g*. the value of t_{99} represents the time necessary to reach 99 percent of the full-scale output. The time which has elapsed until 63 percent of the final value is reached is called the *time constant*. Response time is a function of the kinetics of the reaction that takes place on the electrode surface and it increases as the concentration of the analyte decreases.
- **Selectivity:** Selectivity is related to the accuracy and precision of the analyte measurements in the presence of the inferring substances. Two different classes of

inferring substances affect the response of amperometric sensors. Substances whose response is similar to the analyte and those that interact with the detected compound. Both, mixed solution and separate solution methods can be used for the determination of amperometric selectivity coefficients. In the mixed solution method, selectivity is expressed as the ratio of the signal output with the analyte alone and with the interfering substance alone at the same concentration as that of the analyte. In the separate solution method, interfering substances are added, at their expected concentration, into the measuring cell, already containing the analyte concentration at the mid-range of its expected value. Selectivity is then expressed as the percentage of variation of the biosensor response.

- **Reproducibility:** is a measure of the scatter or the drift in a series of observations or results performed over a period of time. It is generally determined for the analyte concentrations within the usable range.
- ***Stability*:** the ability of the amperometric biosensor to maintain its performance for a certain period of time. As a measure of stability, drift values are used, *e.g*. the signal variation for zero concentration.
- ***Lifetime (t_L)***: the length of time over which the amperometric biosensor will operate, i. e. the storage or operational time necessary for the sensitivity, within the linear concentration range, to decrease by a factor of 10% (t_L10) or 50% (t_L50). The maximum storage time (*shelf life*) must be distinguished from the maximum *operating life*. The latter can be specified either for continuous operation or for repeated on-off cycles.

2.6.2. Laccase (EC 1.10.3.2)

Amperometric biosensors employ redox enzymes as recognition elements. Enzymes are biocatalysts with an extremely high selectivity. They consist of protein molecules with a molecular mass between 10^4 to 10^5 Da. Enzymes are classified by the NC-IUBMB (*Nomenclature Committee of the International Union of Biochemistry and Molecular Biology*) by EC number, which classifies them according to their class and with the reaction they catalyse (Patel, 2002).

In an analytical process, enzymes are used for specific estimation of the corresponding substrates and they provide a significant amplification system for the sensitive detection of a substrate. Enzymatic biosensors utilize specific enzymes for the capture and catalytic generation of the product, which is directly determined by using a range of transducers, however, a majority of enzyme based biosensors employ the amperometric transduction method (Pejcic and Marco, 2006).

Enzymes are divided into six major classes according to their function. These are oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Among the enzymes commercially available, oxidoreductases are the most often used in biosensor applications because of their electron transferring properties during catalysis. This type of enzyme offers the advantages of being stable, and in some situations does not require coenzymes or cofactors (Mello and Kubota, 2002). Oxidoreductases can be classified into four groups; oxidases, dehydrogenases, peroxidases and oxygenases. An enzyme bellonging to the oxidase group was used in this work and will be described in the following paragraphs. Oxidases are the enzymes capable of transfering hydrogen from a substrate to molecular oxygen. Oxidases are also divided into two groups based on the product formed during catalysis. These are water producing (copper containing) and hydrogen peroxide producing oxidases.

The majority of biosensors developed for the detection/quantification of phenolic compounds in food/beverage have been enzyme-based amperometric biosensors. Phenolic compounds are good substrates for oxidases enzymes, therefore biosensors modified with tyrosinase, laccase, and peroxidase, have been developed for the detection of phenolic compounds since they can act as electron donor for these enzymes. The generic name phenoloxidase (PO) and polyphenoloxidase (PPO) have been utilised in many publications to indicate both laccase and tyrosinase enzymes.

Laccase (benzenediol oxygen oxidoreductase, EC 1.10.3.2) catalyses the four-electron reduction of oxygen directly to water (without the intermediate formation of hydrogen peroxide) with four sequential one-electron oxidations of substrate, such as ortho- and paradiphenols, aminophenols, polyphenols, polyamines, lignins and aryl diamine as well as some inorganic compounds (Haghighi *et al.,* 2003; Piontek *et al.*, 2002).

Laccases are found under different forms in nature. Among the different laccases, the enzymes isolated from *Trametes* strains are generally more stable (Ncanana, et al. 2007). Laccase obtained from fungus*Trametes versicolor* (TvLac) was the enzyme used in the present experimental work.

TvLac is a monomer organised in three sequentially arranged domains, with the approximate dimensions of 65 x 55 x 45 Å with a molecular mass of 70 kDa. The spectroscopic studies and X-ray crystallography reveals that the catalytic unit of the enzyme consists of four copper ions classified into Type 1 (T1), Type 2 (T2), and two Type 3 (T3) ions. The T1 copper is located about 6.5 Å below the surface of the enzyme. The T2 and T3 centers are located close together and form a trinuclear copper center. The trinuclear copper center T2/T3 of laccase is located about 12 Å deep within the molecule. The T1 copper is connected to the trinuclear T2/T3 site by a histidine-cysteine-histidine tripeptide. Figure 11 shows the three dimensional structure of TvLac (Piontek *et al*., 2002; Ivnitski and Atanassov 2007).

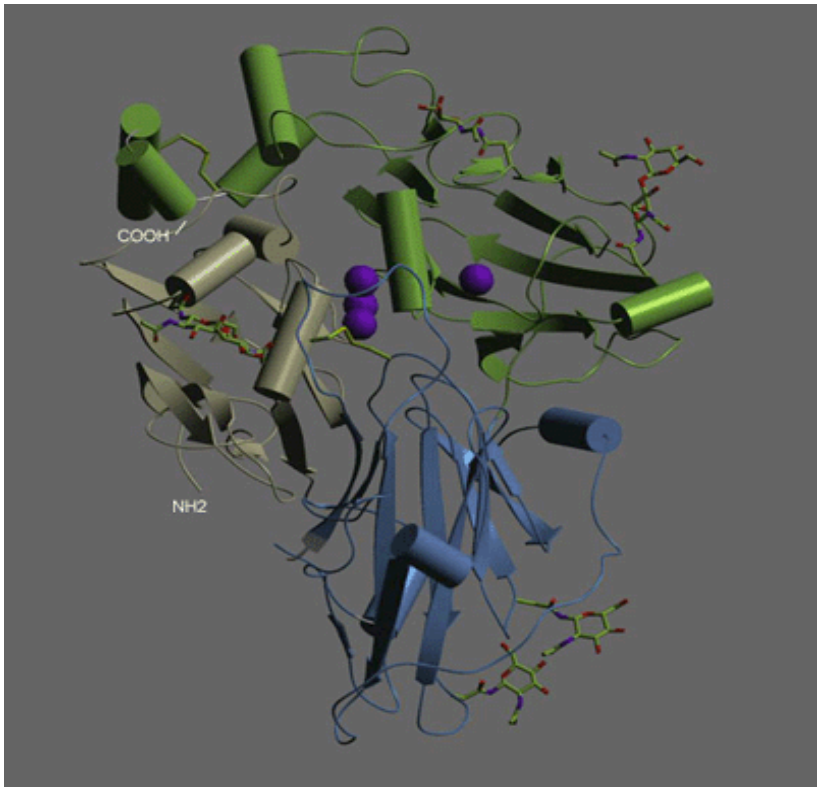


Figure 11. Diagram of the structure of laccase *Trametes versicolor*. The three domains are shown in different colors. The gray part is the N-terminal and C-terminal green. The copper atoms are blue spheres and the portion of carbohydrates and disulfide bonds are included as *stick models.* The T1 site is the isolated sphere (*adapted from* Piontek et al., 2002).

The accepted mechanism for laccase suggests that intramolecular electrons are transferred from the donor group to the acceptor via a hopping mechanism. The laccase enzyme oxidizes substrates by removing one electron per time and generates free radicals which can be polymerised. The enzyme stores electrons of individual oxidation reactions and in its totally reduced state contains a total of four electrons, thus, the enzyme can transfer these electrons to molecular oxygen to form water (Figure 12). *i. e.* the copper ions in the active site of the laccase, provide electron transfer mechanism by switching their oxidation states between Cu(II) and Cu(I). The function of the T1 center is to provide the long-range intramolecular electron transfer from the substrate to the T2/T3 redox copper center. The T2/T3 copper center plays a key role in the reduction of oxygen. Between the two T3 coppers there is an oxygen ligand, either OH^- or O^{-2}, which coordinates with the T2 and T3 copper ions. The solvent and oxygen

have access to the T2/T3 center through two channels, and finally, molecular oxygen is reduced to water (Ivnitski and Atanassov, 2007).

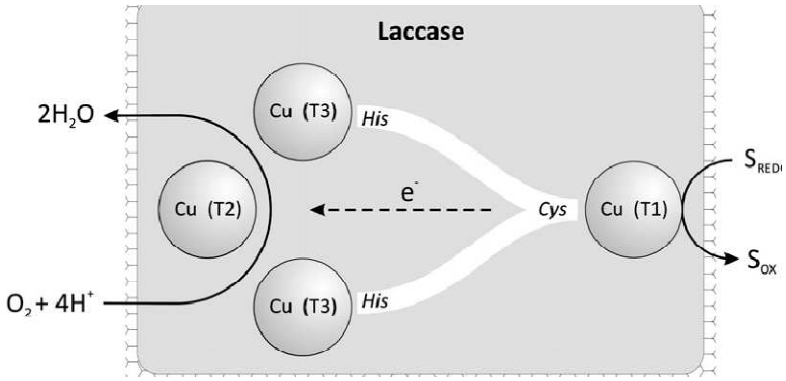


Figure 12. Catalytic mechanism of laccases (*adapted from* Tortolini *et al*, 2010)**.**

The key characteristic of laccase is the standard redox potentials of the T1 site. The values of this redox potential in different laccases have been found to be between 430 and 790 mV vs. the normal hydrogen electrode (NHE) as determined using potentiometric titrations with redox mediators (Shleev *et al*. 2005). *Trametes versicolor* laccase presents a high redox potential l, the value of the redox potential of the T1 Cu-site being 780 mV versus normal hydrogen electrode (NHE), and the T2 Cu-site being about 400 mV versus NHE (Wang *et al*., 2009).

It has been shown that the T1 site is the primary centre, at which electrons from reducing substrates are accepted. Moreover, the catalytic efficiency (kcat/Km) for the majority of the aromatic reducing substrates depends on the redox potential of the T1 copper, which makes laccases with a high redox potential of the T1 site of special interest in biotechnology (Shleev *et al*. 2005).

Laccase (*e.g.*TvLac) possess many attractive properties for the development of amperometric biosensors:

- It is commercial available;
- Within the enzymatic and the electrochemical procedures (analyte regeneration between an electrode and an enzyme) an amplification of the signal can be observed and;
- The broad specificity of the phenolic substrates enables laccase to be developed as a biosensor for the determination of total phenolics (Quan, *et al.* 2004).

2.6.3. Laccase immobilisation

As it is already mentioned at section 2.6.1, a biosensor is developed when the immobilised biological recognition material is in close contact with a physical transducer, However immobilised enzymes are affected by the surroundings in which they operate. The characteristics of an amperometric biosensor are influenced by different effects (Thévenot *et al*., 1999; Gründler, 2007):

- changes in the conformation of the enzyme by the immobilisation process generally leads to a reduction of the affinity to the substrate (increase of K_m), and also to an inactivation of a part of the enzyme or the whole enzyme (decrease of V_{max});
- effects imposed by the matrix (*i.e.* effects of charge, hydrophobicity and matrix structure) can also lead to a change of K_m or V_{max}, and;
- diffusion effects will result in concentration differences within the enzyme' matrix (internal diffusion restriction) as well as within the boundary layer bulk solution/biosensor and a semi-permeable membrane (external diffusion restriction).

For reuse, good sensitivity and faster response, enzymes are immobilised onto the sensor surface or to a support material. The enzymes are easily handable due to the immobilisation and usually the stability of the enzyme is increased. In several studies it is reported that laccase in its immobilised form is more stable than laccase in solution (Fei, *et al.,* 2007). The purpose of any immobilisation method is to retain maximum activity of the biological component on the

surface of the transducer. The selection of an appropriate immobilisation method depends on the nature of the biological element, type of the transducer used, physicochemical properties of the analyte and operating conditions for the biosensor. Several methods can be used for immobilising the enzyme onto the electrode surface including adsorption, covalent attachment, cross-linking, entrapment and encapsulation. These methods have been extensively reviewed elsewhere (Guilbault, 1984; Turner *et al*., 1987, Mosbach, 1988; Cass, 1990; Göpel, 1991; Blum, 1991). The immobilisation method adopted should not only increase the stability of the bioreceptor but also retain its bio-recognition property to extend the useful life of the sensor.

Immobilisation of laccase for the construction of electrochemical biosensors has been carried out on different electrode materials: self-assembled monolayer on gold (Gupta *et al.*, 2002), glassy carbon (Gamella et al., 2006), polyethersulfone membranes (Gomes *et al*, 2004), carbon fibres (Freire *et al.,* 2002), graphite (Haghighi *et al.,* 2003), platinum (Quan et al., 2004), screen printed (Ibarra-Escutia *et al.*, 2010) among others.

The covalent immobilisation of the TvLac on modified PES membranes (PES/Tvlac) in order to prepare the phenolics' biosensor was used in experimental work (Gomes *et al*, 2004). It is an easy, rapid and effective method of immobilisation.

2.6.4. Mechanism of the reactions on the laccase-based biosensor for phenolic compounds detection

Laccase biosensor advantage with respect to peroxidase biosensors in that it does not require hydrogen peroxide to oxidise phenolic substrates. Biosensors based on tyrosinase suffer from low enzyme stability and significant inhibition of the enzyme by reaction products, which makes laccase an alternative, strong candidate for application in biosensor for the determination of phenolic compounds (Gupta *et al.*, 2002; Gamella *et al*., 2006). This enzyme catalyses the oxidation of phenolic compounds (PC) by molecular oxygen according to the reaction,

$$AH_2 + {}^1/_2O_2 \xrightarrow{\text{laccase}} A + H_2O$$

where $AH_2 = PC_{RED}$ and $A = PC_{OX}$, reduced and oxidised states of phenolic compounds.

As seen in section 2.6.2, laccases can catalyse the oxidation of *o*-, *m*-, and *p*-benzenediols and phenol in the presence of molecular oxygen to *o*-, *m*-, *p*-quinones or radical species and does not require hydrogen peroxide as a cosubstrate or any cofactors for the catalytic reaction (Gamella *et al*., 2006). Schematical representation of the mechanism of the reactions on laccase biosensor is given in Figure 13. Immobilised laccase catalyses the oxidation of phenolic compound (PC_{RED}) in solution to the corresponding o-quinone (PC_{OX}), whereby the enzyme is oxidized back to its native form by molecular oxygen*. i.e*. while this transformation occurs, molecular oxygen is simultaneously reduced on the electrode surface producing water according to a direct four-electron mechanism as shown in the following reaction,

$$O_2 + 4H^+ + 4e^- \xrightarrow{\text{laccase}} 2H_2O$$

The product of enzymatic oxidation (o-quinone) is subsequently reduced on the working electrode at the appropriate potentials. The resulting reduction current (I_R) is proportional to the concentration of PC, thus, I_R is used as the analytical response for PC' determination.

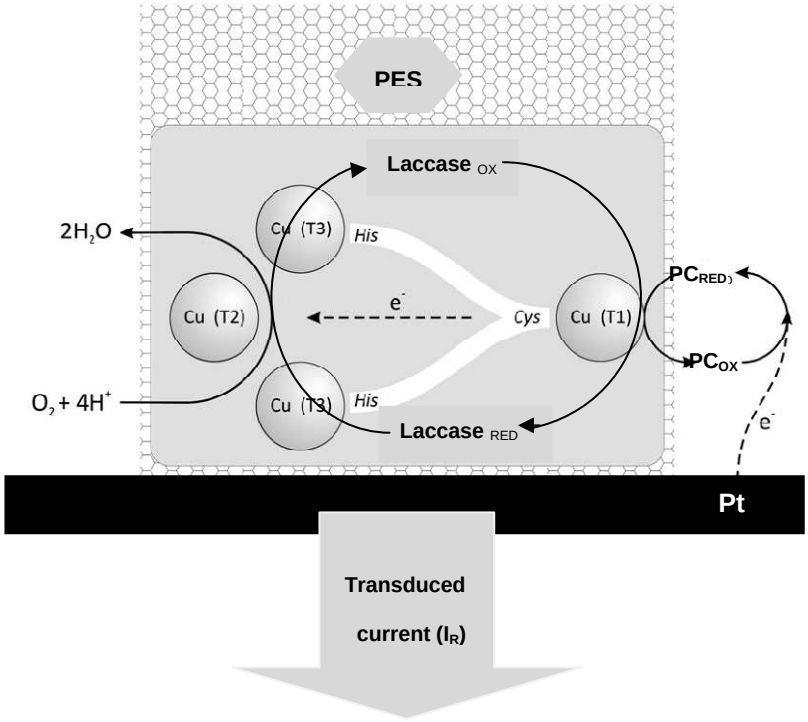


Figure 13. Mechanism of the reactions on the laccase biosensor; PC: phenolic compound; PES: derivatised polyethersulfone membrane (*adapted from* Tortolini *et al.*, 2010) .

Laccase enzyme is able to catalyse direct (mediatorless) electron transfer, concept introduced in section 2.6.1 (last paragraph). The combination of the electrochemical detection principle and the recycling of the PC (analyte) between the immobilized laccase and electrode is performed by bioelectrocatalytic recycling (or electrochemical substrate regeneration), which is one of the most applied amplification principles in the development of high sensitive laccase electrodes.

The use of the electrochemical reduction of generated o-quinones as detection reaction for the quantification of PC gives at least two advantages over direct electrochemical oxidation of PC, as seen in section 2.5.3.2:

- The reaction inactivation by polymerisation is hindered by the electrochemical removal of quinone;
- The low applied potential minimises the influence of different sample-matrix interferences.

2.6.5 TvLac-based biosensor for polyphenolic content of wines and teas

Caffeic acid (3,4-dihydroxycinnamic acid) is a phenolic acid, as described in section 2.3.1.2, and was used as the standard compound for the bioelectrochemical polyphenolic index' (BPI) determination in wine and tea. Thus, the results were expressed as caffeic acid equivalents (CAE) concentration.

In fact, gallic acid has been used as standard compound to express the results obtained by application of the Folin-Ciocalteu method. However, in laccase biosensing determination, caffeic acid exhibits a considerably higher relative sensitivity than gallic acid at laccase biosensors (Gamella et al., 2006; Montereali et al., 2010), as shown below in Table 6, being also used to express the polyphenolic content in samples. Moreover, caffeic acid is one of the phenolic compounds present in a high amount in wine and tea, being used as reference by some authors, when measurement of the total content of phenolics of the sample is undertaken. Caffeic acid has been studied by several researchers (Campanella *et al*., 2004; Jarosz-Wilkołazka *et al.*, 2004; Carralero *et al*., 2005; Gamella *et al.,* 2006), who have found out that it would be the preferred substrate to be used for this purpose among other polyphenols analysed, being redox recycled between the immobilised laccase (enzymatic oxidation reaction) and the electrode (electrochemical reduction reaction). *i.e.* in a laccase reaction caffeic acid is subject to one-electron oxidation, giving rise to an aryloxyradical (semiquinone), which can be converted to a quinone in the second stage of oxidation. Ortho-quinone derived from caffeic acid can be chemically reduced to caffeic acid as shown in Figure 14.

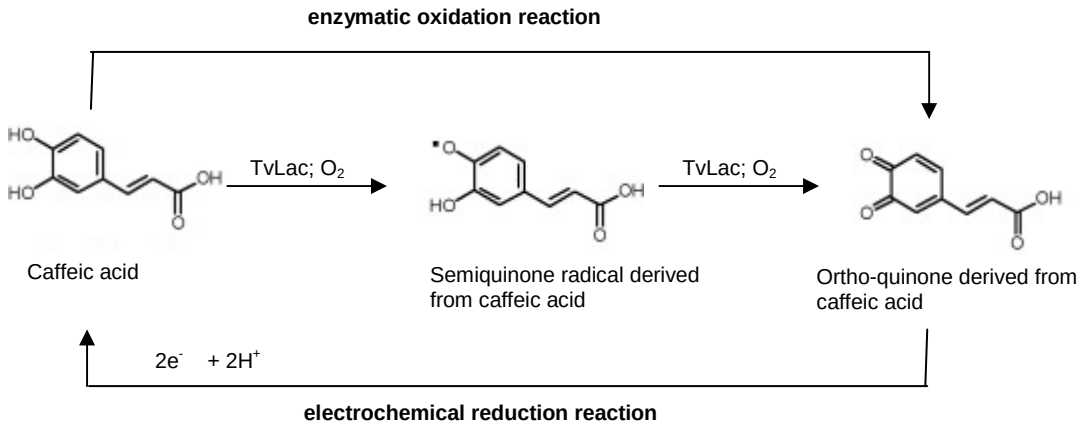


Figure 14. Oxidation of caffeic acid and the corresponding electrochemical reduction (redox recycling).

The detection principle applied in TvLac biosensor used in experimental work was the enzymatic oxidation followed by electrochemical regeneration, as seen above. The setup for TvLac-based biosensor used in experimental work is shown in Figure 15. The experiments were carried out at a constant potential, E_{apl}, (+0.1 V *vs* Ag/AgCl), and the current response corresponding to the reduction of enzymatically produced o-quinone was recorded as function of time as shown in Figure 15 c. Comparison between the signal due the addition of the sample and that due to the caffeic acid (standard compound) by using an equation that was obtained from the standard caffeic acid plot allowed the phenolics content of the sample to be determined as caffeic acid equivalents (CAE), and then expressed in mg L^{-1} of caffeic acid

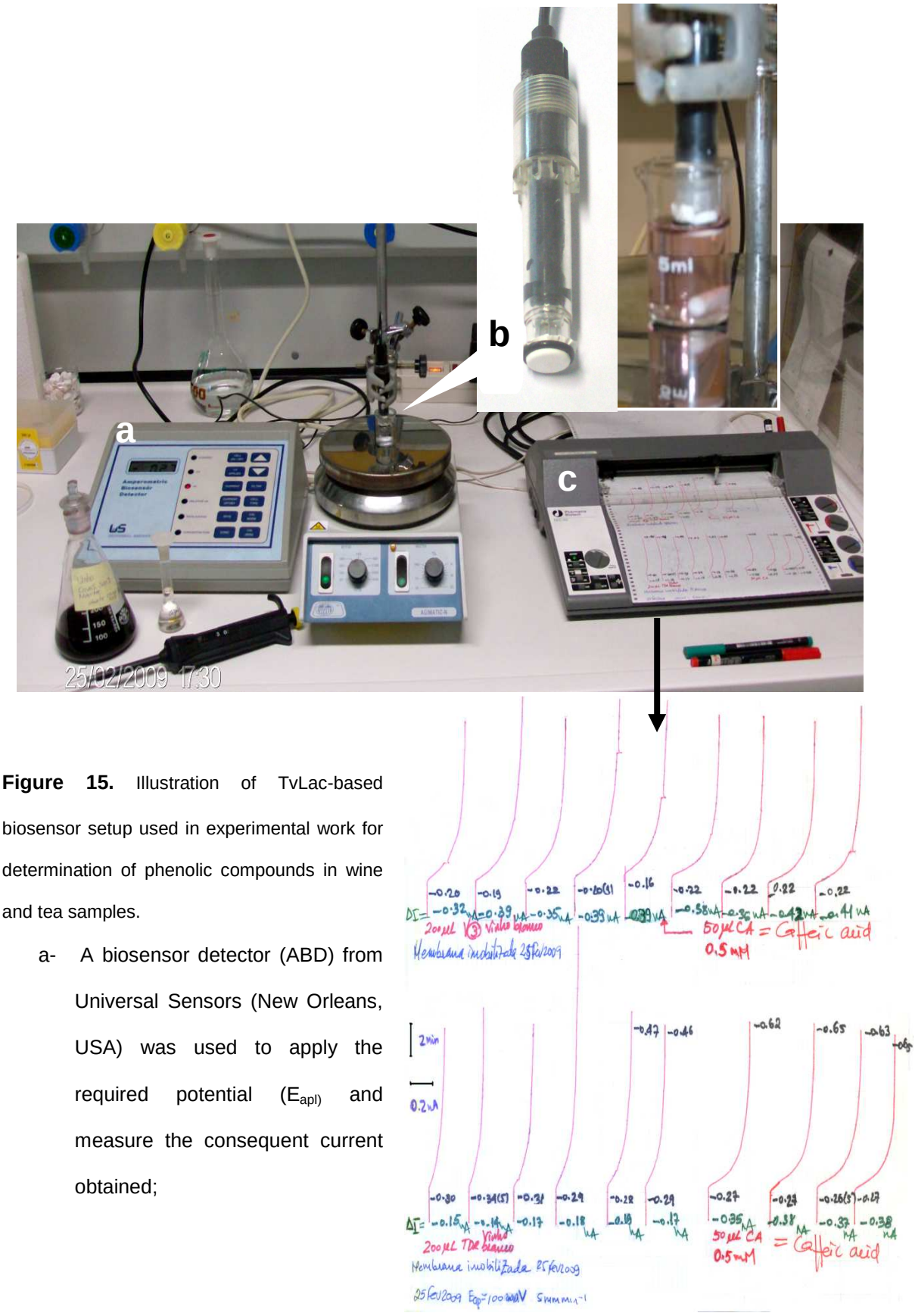


Figure 15. Illustration of TvLac-based biosensor setup used in experimental work for determination of phenolic compounds in wine and tea samples.

a- A biosensor detector (ABD) from Universal Sensors (New Orleans, USA) was used to apply the required potential (E_{apl}) and measure the consequent current obtained;

b- An electrode base sensor, Pt–Ag, AgCl from Universal Sensors (New Orleans, LA). The electrode base system consisted of platinum wires 0.3 mm diameter and a silver, silver chloride helix which were contained in an electrode plastic cap closed on one end by an internal dialysis membrane. The bioelectrode was prepared by attaching the enzyme-modified membrane (PES/Tvlac) to the electrode base system, with an o-ring;

c- The transients of current versus time were recorded on a Pharmacia Biotech recorder REC102.

The response of the TvLac- based biosensor towards caffeic acid in tartarate buffer at pH 3.5, a detection potencial (E_{ap}) of + 0.1 V (*vs* Ag/AgCl) presented the following analytical characteristics (Júnior and Rebelo, 2008):

- Sensitivity: 0.102 mA M^{-1},
- Reproducibility: RSD = 1.9% (n=5),
- Linear range: 5 to 35 × 10^{-6} M and,
- Limit of detection (LOD): 8.8 ×10^{-7}M.
- Lifetime: 71.4% of the initial activity retained after 24 days of storage (dry at 4 $^{\circ}$C).

The presentation of the chapter 2, literature review, of this thesis ends with a list (Table 6) for comparison of the analytical performance of some polyphenoloxidase and peroxidase – based biosensors for the determination of phenolic compounds in wine and tea samples, which were published during the last decade. Characteristics such as the type of electrode and immobilisation method, detection potential, sensitivity, limit of detection and useful lifetime are referred.

Table 6. The analytical characteristics of some amperometric biosensors for the determination of phenolic compounds in wine and tea samples.

Biosensor (enzyme + electrode)	Immobilisation	Sample	$E_{ap,}$ V	substrate	Sensitivity (mA M^{-1})	Linear range (M)	LOD (M)	Lifetime	Reference
Laccase (TvLac) Screen-printed graphite electrodes	Entrapment in polyvinyl alcohol film	Teas	-0.3 vs Ag/AgCl	Caffeic acid	24.91	(50-130)x10^{-6}	5.24x10^{-5}	6 months	Ibarra-Escutia *et al.*, 2010
Laccase (TvLac) graphite screen printed electrodes modified with ferrocene	sol–gel matrix of diglycerysilane (DGS)	wines	+0.050 vs Ag/AgCl	Caffeic acid Gallic acid	6.26 0.10	---	6x10^{-6} 3.8x10^{-4}	5 days	Montereali *et al.*, 2010
Laccase (TvLac) Carbon nanotubes screen-printed electrodes	Chemical and physical entrapment with polyazetidine pre-polymer	Wines	-0.1 vs Ag/AgCl	Gallic acid	1.53	(0.6-100)x10^{-6}	0.6	10 days	Fusco *et al.*, 2010
Tyrosinase graphite screen printed electrodes modified with ferrocene	sol–gel matrix of diglycerysilane (DGS)	wines	+0.050 vs Ag/AgCl	Caffeic acid Gallic acid	0.85 0.17	---	7.8x10^{-5} 5.8 x10^{-5}	5 days	Montereali *et al.*, 2010
Tyrosinase Glassy carbon electrode	Cross-linking with glutaraldehyde	Wines	-0.2 vs Ag/AgCl	Caffeic acid Gallic acid	102 16	(0.04-5.88)x10^{-7} (0.18-47)x10^{-7}	4.23x10^{-9} 1.53x10^{-8}	4 days	Gamella et al., 2006
Tyrosinase Clark oxygen electrode	Cross-linking with glutaraldehyde	Teas	---	(+) catechin	---	(1-9)x10^{-7}	---	6 days	Abhijith *et al.*, 2007

Table 6. The analytical characteristics of some amperometric biosensors for the determination of phenolic compounds in wine and tea samples (cont.).

Biosensor (enzyme + electrode)	Immobilisation	Sample	E_{apl}, V	Standard substrate	Sensitivity (mA M^{-1})	Linear range (M)	LOD (M)	Lifetime	Reference
Tyrosinase Glassy carbon electrode	Cross-linking with glutaraldehyde	Wines	-0.1 vs Ag/AgCl	Caffeic acid	14	(2-2009)x10^{-6}	6.6x10^{-7}	18 days	Carralero et al., 2005
HRP (peroxidase) Carbon paste electrode modified with ferrocenearbon	Entrapment in the composite	Wines and teas		(+) catechin	0.76	(0.3-15)x10^{-6}	5.17x10^{-7}	15 days	Kong et al., 2001
PBHR (peroxidise from Brassica napus) Composite carbon nanotubes electrodes	Entrapment in the composite	Wines and teas		t-Resveratrol Caffeic acid	1.54 0.92	(0.30-306)x10^{-6} (0.40-406)x10^{-6}	1.35x10^{-7} 1.18x10^{-7}	5 days	Granero et al., 2010

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CHAPTER 3

ARTICLES

ARTICLE I

Evaluating the antioxidant capacity of wines: a laccase-based biosensor approach

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Eur Food Res Technol 231(2) (2010) 303-308

DOI 10.1007/s00217-010-1280-0

ABSTRACT

Polyphenolic compounds present in wine have a high antioxidant capacity providing a protective effect on human health. There is a worldwide agreement on this assertion. However there is not agreement on the method of assessing the antioxidant capacity of wines. The purpose of this study was to emphasize the ability of the laccase biosensor developed by our group to evaluate the antioxidant capacity of several types of wine in relation to their polyphenolic content. The values of total antioxidant activity of the investigated wine samples were well correlated with the corresponding bioelectrochemical polyphenol estimated values, the correlation coefficient obtained being $r = 0.9795$ ($p < 0.05$). The linear relationship between total antioxidant activity and bioelectrochemical polyphenol index determined by our laccase biosensor enabled the calculation of the TEAC (Trolox Equivalent Antioxidant Capacity) of a wine from its bioelectrochemical polyphenol index.

Keywords: Laccase-biosensor; caffeic acid; polyphenols; antioxidant.

1. INTRODUCTION

Antioxidants have been the subject of many epidemiological researches over the last decade. A large number of studies have shown that moderate and regular consumption of wine, especially red wine, appears to be strongly related to prevention of several pathologies such as cardiovascular and neurological diseases, cancers and cell mutation associated with aging [1].

The phenolic compounds present in wines are widely known to have a high antioxidant activity, providing the protective activity against those diseases [2]. On the other hand, these compounds belong to a class of phytochemicals that contributes to the quality of wines, affecting their organoleptic characteristics, in particular color and astringency. Wine phenolic composition varies considerably in different types of wine, depending on the grape variety and vinification conditions [3].

A wide range of studies has shown that phenolic compounds play a role as antioxidants through different mechanisms of action, such as free-radical scavengers, metal-chelating agents, inhibition of lipoprotein oxidation, quenching of reactive oxygen species and inhibition of oxidative enzymes [4, 5].

For all the above reasons, it is of great interest to evaluate the antioxidant capacity of the wines in relation to their polyphenolic content. Studies have been published on red and white wines, and the antioxidant properties of wines have been correlated with their polyphenolic contents [6 –15].

There are a number of different analytical methods to determine the antioxidant activity of wine [16]. One of the most commonly used is the 2,2-azinobis(3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS) assay, based on the generation of the highly stable chromophoric cation radical of ABTS. Its decolorization by an antioxidant in the reaction medium reflects the amount of cation radical ABTS that has been scavenged after a fixed period of time. The antioxidant activity is compared to that of trolox as the standard (TEAC) [17]

Several methods to quantify total phenols and polyphenols have been described in the literature, HPLC and GC being very common techniques used for this purpose [18]. The Folin–Ciocalteu method is widely employed in the wine industry. This method measures a sample reducing capacity, which does not, necessarily, reflect an absolute measurement of the total phenolic content [19, 20].

Amperometric biosensors using enzymes such as tyrosinase [13, 15], laccase [14, 21–23] and peroxidase [24] have been proposed as attractive alternative analytical methods for the detection of polyphenolic compounds. Electrochemical biosensors have also been used as tools for estimating the antioxidant capacity [25, 26].

In a previous article [23], the development of a laccase-based biosensor for the polyphenolic content of wine determination was presented. Basically, the polyphenolic content of the wine was related to the amperometric signal due to the reduction of the quinone obtained as a product of the enzymatic oxidation of polyphenolic compounds. Those reactions are summarised on Figure 1.

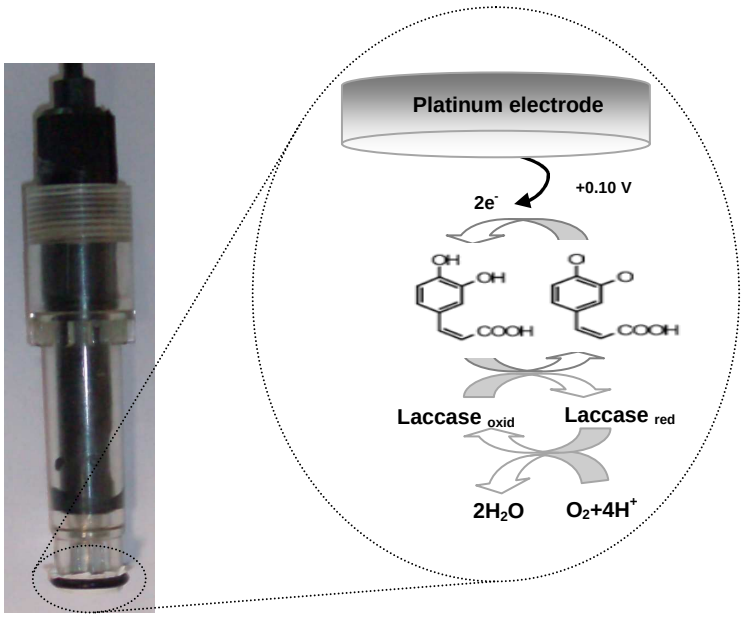

Figure 1. Scheme illustrating the oxidation of caffeic acid by laccase and reduction of the formed o-quinone on the biosensor surface.

The aim of this work was to evaluate the caffeic acid equivalent of different Portuguese wines (white, rosé and red) using the laccase-based biosensor, to determine the antioxidant power of the same wines by the TEAC methodology and to obtain the corresponding correlation.

2. MATERIALS AND METHODS

2.1. Reagents and solutions

Trametes versicolor laccase (E.C.1.10.3.2, 23.1 U mg^{-1}) was from Fluka (Steinheim, Germany). Trans-3,4- dihydroxycinnamic acid (caffeic acid), 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid (trolox), Folin–Ciocalteu's phenol reagent (FC reagent), disodium hydrogen phosphate (anhydrous) (Na2HPO4) and potassium dihydrogen phosphate (anhydrous) (KH2PO4), with purity superior to 99.9% and 99.7%, respectively, were obtained from Sigma–Aldrich (St. Louis, MO). 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in the crystallized diammonium salt form was from Roche (Mannheim, Germany). Tartaric acid, NaCl with purity greater than 99.8% and KOH were obtained from Riedel–deHae¨n (Seelze, Germany). Potassium persulfate (K2S2O8) purity superior to 99% and KCl purity superior to 99.5% were from Merck (Darmstadt, Germany). Absolute ethanol, density 1.790 kg/L, and sodium carbonate were obtained from Panreac Quimica AS (Barcelona, Spain). Caffeic acid solutions were freshly prepared at a concentration of 0.5 mM in tartrate buffer pH 3.5, on the day of the experiment. All aqueous solutions were prepared using water purified with a Milli-Q A10 system (Millipore). Polyethersulfone membranes (Ultrabind US450 0.45 lm) were from Gelman.

2.2. Wines

A total of 17 commercially available Portuguese wine samples were analysed: white (n = 5), rosé (n = 3) and red (n = 9) wines. Some of them were bought in a local shop, and others were gently offered by SOGRAPE, S.A.

2.3. Equipment

Amperometric determination was done using a 4208 electrode base sensor, Pt–Ag, AgCl from Universal Sensors (New Orleans, LA). An amperometric biosensor detector (ABD) from Universal Sensors (New Orleans, USA) was used to apply the required potential and measure the consequent current obtained. The transients of current versus time were recorded on a Pharmacia Biotech recorder REC102.

Spectrophotometric measurements were taken on a UV–Vis double beam Shimadzu UV-1603 spectrometer (Shimadzu, Kyoto, Japan) connected to a PC (UVPC® Personal Spectroscopy Software).

2.4. Determination of bioelectrochemical polyphenolic index (BPI) using the laccase-based biosensor

Laccase-based Biosensor preparation was performed as follows: the membrane was cut in the form of disks of 18 mm diameter and contained 9.2 U of laccase. The membranes with the immobilised enzyme were applied to a US electrode system base. The electrode base system consists of platinum wires 0.3 mm diameter and a silver, silver chloride helix, which were contained in an electrode plastic cap closed on one end by an internal dialysis membrane. The external membrane containing the immobilised enzyme was juxtaposed to the internal membrane. Both were held tightly to the electrode plastic cap by an Oring.

The biosensor thus prepared was dipped in 5 mL of the buffer (tartrate at pH 3.5), and injections of standard (50 µL) (caffeic acid 0.5 mM in tartrate buffer pH 3.5) were done, under magnetic stirring, at 300 rpm. The experiments were made at a constant potential (100 mV versus Ag/AgCl). The current response was recorded as function of time. The biosensor was used to measure the relevant cumulative effect of the polyphenolic content of wines without pretreatment. Wine samples (200 µL) were injected in 5 mL of the buffer (tartrate at pH 3.5),

following the same procedure as for the standard. The results were expressed as milligrams of Caffeic Acid Equivalents (CAE) per litre of wine, averaged from triplicates.

2.5. Determination of total phenolic content (TPC) using Folin–Ciocalteu method

The total phenolic content of wines was determined spectrophotometrically according to the Folin–Ciocalteu (FC) colorimetric assay, with the microscale protocol [27], which is based on the method reported in [19]. The reaction took place directly in an Eppendorf tube. For this purpose, 20 µL of wine sample (all red wines were diluted 100x, rose´ and all white wines were diluted 109 with water) was pipetted into an Eppendorf, and 1.58 mL water was added, followed by 100 µL of the FC reagent. The mixture was allowed to stand for 1 min, and then 300 µL of the sodium carbonate solution (20% (w/v)) was added. The reaction mixture was kept in the dark for 2 h, and its absorbance, measured at 765 nm against the blank. The phenolic content in wine was evaluated from the generated absorbance value by the interpolation into calibration plots constructed for caffeic acid. The results (averaged from triplicates) were expressed as milligrams of Caffeic Acid Equivalents (CAE) per litre of wine.

2.6. Determination of total antioxidant activity (TAA) using ABTS assay

The total antioxidant activity of the wines was determined according to the method described in [28]. The assay is based on the capacity of a sample to inhibit the ABTS radical cation compared with a reference antioxidant standard (Trolox). The reaction took place directly in the measuring cuvette. For this purpose, 10 µL of the wine sample was pipetted into separate cuvettes, 1 mL of the diluted ABTS radical cation solution was added, and the absorbance at 415 nm was measured during 2 min [8]. This was compared to a blank where 1 mL of the diluted ABTS cation radical solution was added to 10 µL of the solvent. All red wines were diluted 100x, and rosé and all white wines were diluted 10x with 20% ethanol aqueous solution. The results (averaged from triplicates) were expressed as Trolox Equivalent Antioxidant Capacity (TEAC, mmol L^{-1}).

2.7. Statistical analysis

The data were expressed as the mean of triplicates ± standard deviation (SD). Analysis of variance (ANOVA) at level $p < 0.05$ and linear correlations test were carried out using the SigmaStat 3.5 (Systat Software Inc.).

3. RESULTS AND DISCUSSION

The caffeic acid equivalent measured by the present laccase-based biosensor (BPI), the total phenolic content according to the Folin–Ciocalteu spectrophotometric method (TPC) and the total antioxidant activity (TAA) of 17 Portuguese commercial wines, 9 red, 3 rosé and 5 white wines, were analysed and compared. The results of BPI, TPC and TAA are shown on Table 1.

3.1. Phenolic content of wines

The bioelectrochemical polyphenolic index (BPI) obtained from our laccase-based biosensor pointed to a wide range of phenolic content in the studied wines, as evidenced on Table 1. The amount of phenolics varied considerably in different types of wines. As expected, it decreased in the order: red > rosé > white wines, according that found in the literature [13–15] for biosensing determination. Red wines contained high concentration of phenolics, ranging from 61.1 to 82.7 CAE mg L^{-1}, with an average of 69.2 CAE mg L^{-1}. For rosé wines, BPI ranged from 27.9 to 31.0 CAE mg L^{-1}, averaging 29.8 CAE mg L^{-1}. The values for white wines were statistically different from those of rosé wines, ranging from 7.0 to 12.3 CAE mg L^{-1}, with an average of 10.0 CAE mg L^{-1}.

Table 1. Bioelectrochemical polyphenolic index (BPI), total phenolic content (TPC) and total Antioxidant activity (TAA) of wines.

Wine sample identification	BPI (CAE, mg L^{-1})[b]	TPC (CAE, mg L^{-1})[b]	TAA (TEAC, mmol L^{-1})[c]
R1	61.1 ± 2.3	2,585 ± 50	21.45 ± 0.16
R2	65.9 ± 4.7	2,385 ± 57	21.11 ± 0.16
R3	68.1 ± 0.7	2,268 ± 29	21.47 ± 0.13
R4	68.7 ± 4.8	2,227 ± 116	18.75 ± 0.24
R5	68.6 ± 1.6	2,501 ± 104	22.72 ± 0.21
R6	82.7 ± 0.9	2,727 ± 151	25.45 ± 0.22
R7	75.3 ± 4.8	2,205 ± 50	21.23 ± 0.14
R8	66.5 ± 2.1	2,193 ± 165	18.10 ± 0.32
R9	65.5 ± 1.9	2,160 ± 71	18.17 ± 0.34
RS1	30.5 ± 1.3	624 ± 38	3.90 ± 0.04
RS2	27.9 ± 0.8	544 ± 42	4.63 ± 0.02
RS3	31.0 ± 0.3	657 ± 28	4.41 ± 0.03
W1	7.7 ± 0.2	239 ± 18	0.87 ± 0.01
W2	12.3 ± 0.3	257 ± 46	1.27 ± 0.02
W3	10.8 ± 1.4	304 ± 32	1.13 ± 0.01
W4	7.0 ± 1.4	321 ± 64	1.20 ± 0.02
W5	12.0± 0.4	420 ± 38	1.17 ± 0.01

R, red; RS, rosé; W, white wines.

[a] Each value is the mean of triplicates ± standard deviation (SD).

[b] Expressed as mg caffeic acid equivalents (CAE) per L of wine.

[c] Expressed as trolox equivalent antioxidant capacity (TEAC) mmol per L of wine.

These results were in good agreement with those reported [14, 15]. The concentration of phenolics estimated as TPC and shown on Table 1 ranged from 2,160 to 2,727 CAE mg L^{-1} for red wines, the average being 2,361 CAE mg L^{-1}. For rosé wines, TPC ranged from 544 to 657 CAE mg L^{-1}, averaging 608 CAE mg L^{-1}. The values for white wines, ranging from 239 to 420 CAE mg L^{-1}, with an average of 308 CAE mg L^{-1}, were statistically different ($p < 0.05$) from those of rosé wines. The values of the phenolic content, TPC, were determined from the regression equation of the calibration curve ($ABS_{765\ nm} = 0.001\ C_{CA}\ mgL^{-1} - 0.0007$; $r = 0.9986$) and expressed in caffeic acid equivalents. The present work results were consistent with those found in the literature. The very high phenolic content of our red wines was of the same order of magnitude as found for other wines by several authors [6–10, 12, 14, 15, 29–31]. Very different numerical values have been obtained for the phenolic content of wines according to the method that is used in the analysis [15]. Folin–Ciocalteu values for the phenolic content of wine can be higher than values obtained by biosensors [14, 15]. The results depend on the standard phenolic compound taken as biosensing reference [14]. Our finding [23] that laccase biosensor was much more sensitive to caffeic acid than to gallic acid was confirmed by other authors [14] who said that ‘‘laccase biosensor can be used to estimate the polyphenol index in wines instead of the Folin–Ciocalteu method, using caffeic acid as the standard phenolic compound’’. If we took gallic acid solution as reference, the BPI numerical values would become closer to the numerical values obtained by the Folin–Ciocalteu method. However, we decided not to do it because the biosensor response to gallic acid was much less sensitive than to caffeic acid. In fact, both methods are comparative methods. They can only give an equivalent value that is always related to a definite polyphenol. That was the reason that made us name our biosensor values as bioelectrochemical polyphenolic ‘‘index’’. The present work is, thus, a pursuing of previous studies extending them to a wider range of Portuguese wines than done before. Furthermore, we measured the antioxidant power of the whole range of wines and found a correlation between BPI and TAA.

3.2. Total antioxidant activity

The free-radical scavenging activity of different wines was estimated by the ABTS method. The TEAC values express the relative ability of hydrogen- or electron-donating antioxidants of a wine sample to scavenge the ABTS radical cation compared to that of trolox. The TEAC value was obtained by interpolating the decrease in absorbance of a corresponding diluted wine sample on the calibration curve ($ABS_{415\ nm} = 0.0029\ C_{trolox}\ \mu M + 0.0037$; $r = 0.9993$), obtaining a concentration of trolox. Thus, the TEAC value of wine expresses the concentration of trolox solution whose antioxidant activity was the same as that of the wine. The results obtained for wines are summarized on Table 1. Red wines had significantly higher TAA than rosé and white wines. The TAA values varied from 18.10 to 25.45 TEAC mmol L^{-1}, averaging 20.94 TEAC mmol L^{-1} for red wines. For rosé wines, 3.90 to 4.63 TEAC mmol L^{-1}, with an average of 4.31 TEAC mmol L^{-1} were found. The values for white wines were statistically different from those of rosé wines, ranging from 0.87 to 1.27 TEAC mmol L^{-1}, with an average of 1.13 TEAC mmol L^{-1}. These results were in agreement with those presented in the literature [8, 11]. TPC and TAA of all studied wines (including rosé wine) were highly correlated, $r = 0.9950$ ($p < 0.05$). This result was in agreement with the claim that the main source of antioxidant activity derives from phenolic compounds in wine, as has been reported in the literature [6–15].

3.3. Correlation between bioelectrochemical polyphenolic index and antioxidant activity

In order to evaluate the total antioxidant activity of the wines by laccase-based biosensor, TAA was plotted against BPI (Figure 2). The corresponding correlation coefficient obtained was r = 0.9795 ($p < 0.05$), clearly indicating that the BPI had good correlation with TAA, the equation being:

$$TEAC_{wine} = 0.343(\pm 0.018)\ CAE_{wine,\ biosensor} - 3.22(\pm 0.96) \qquad \text{Eq. 1}$$

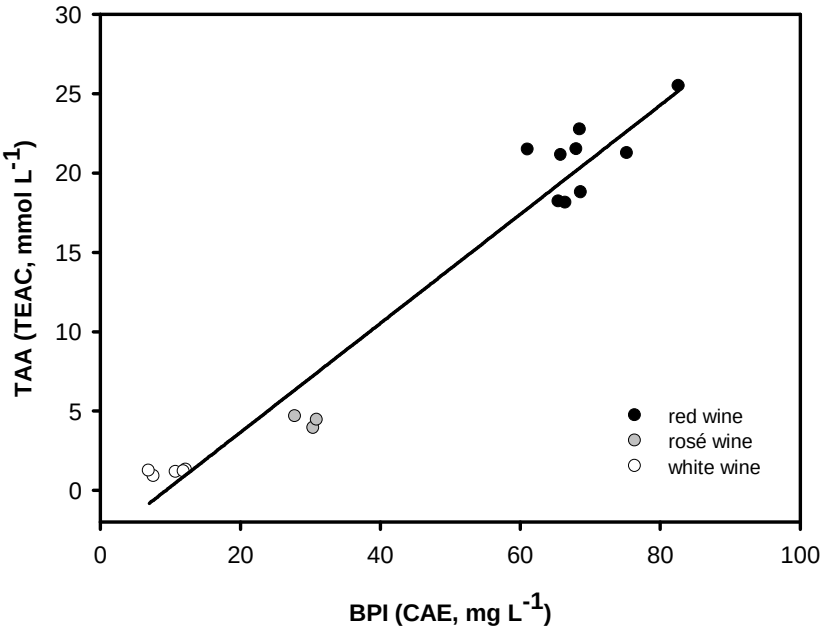

Figure 2. Linear correlation between total antioxidant activity (TAA) and bioelectrochemical polyphenolic content (BPI) of wines ($r=0.9795$, $p<0.05$).

The linear relationship between TAA and BPI thus obtained enabled the calculation of the TEAC of a wine from its caffeic acid equivalent, determined by biosensor, according to a simple expression (Eq. 1). Our laccasebased biosensor was suitable for this determination and exhibits advantages as easy sample preparation, low cost and fast evaluation of wine TAA.

4. CONCLUSIONS

The present work involved the development of a new methodology to evaluate the antioxidant activity of wines, using a laccase-based biosensor developed by our group. In fact, a good correlation was observed between the antioxidant properties of the studied wines (TAA) determined by the ABTS assay and the bioelectrochemical polyphenol index (BPI) obtained at pH 3.5 with the biosensor, taking caffeic acid as the reference solution. The linear relationship between TAA and BPI enabled the calculation of the antioxidant activity of red, rosé and white wines, from its caffeic acid equivalent content, evaluated by the present biosensor. Our laccase-based biosensor methodology has advantages relative to other ones above referred. In fact, it is straightforward to prepare, with its rapid immobilisation of the enzyme on the derivatized polyethersulfone membranes. Moreover, standards and measurements were at pH 3.5, which is a value close to the average pH of wines. The present biosensor, with a disposable membrane, has potential application in the food industry as a bioelectrochemical polyphenol index. This application follows the trend to exploit biosensor technology in areas other than medical diagnostics, where commercial glucose disposable biosensors are currently used.

ACKNOWLEDGEMENTS

We thank Fundacão para a Ciência e a Tecnologia for the grant: FRH/BD/34511/2006 and Financial support from Faculdade de Cieˆncias da Universidade de Lisboa.

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ARTICLE II

Metabisulfite interference in biosensing and Folin-Ciocalteu analysis of polyphenols

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Microchimica Acta **167 (3)** (2009) 253

DOI: 10.1007/s00604-009-0249-9

ABSTRACT

Sodium metabisulfite is one of the forms of sulphurous compounds which is added as preservative in food and drink processing industries. Its interference with different methods of measurements of polyphenols was studied in the present work. Studies involved the reaction of metabisulfite at platinum electrodes either at a constant applied potential: +100 mV vs Ag, AgCl with amperometric detection, or at linear potential sweep, in cyclic voltammetry experiments. In a second stage its influence on measurements of polyphenols which are oxidised by enzymes was checked by the inhibition that metabisulfite caused on caffeic acid oxidation in the presence of free and immobilised laccase. It was found that immobilised laccase was less inhibited than free laccase. A confirmation of the influence of metabisulfite on Folin-Ciocalteu measurements was also done.

Keywords: metabisulfite; laccase; polyphenolic; biosensor.

1. INTRODUCTION

The applicability of electrochemical biosensors to the analysis of antioxidant compounds, including polyphenols, is promising and there is a growing interest in the development of such devices. Nevertheless, it is reckoned that further work is required to avoid and/or take into account the interference problem [1].

Some research is focused on modification of the sensors in order to avoid interferences. A sonogel–carbon electrode, with the laccase in a special immobilisation matrix, was found to be free from interferences when used in beers [2]. However, such as is often the case in other published studies, those authors did not investigate the possibility of the sulfite interference.

Other researchers, acknowledging the chemical complexity, and the presence of complex interferences in food samples, recommended that an “index” could be accepted as an approach to know the “total polyphenolic” content [3].

Ascorbate and sulfite were reported to be important potentially interfering substances on the total polyphenolic content determination by the Folin-Ciocalteu method [4]. Though flow based procedures have claimed to decrease those interferences [5, 6]. An enzymatic method used by Stevanato *et al*. [7] found that there was no interference from ascorbate, but sulfite interfered with the enzymatic method, as well as interfering with the Folin Ciocalteu determination of total polyphenols.

In fact, interferences from substances in real matrices occur, whatever method is used. Though many authors do not do studies on the interference of sulfites and sulphurous species which are used as food preservatives, it is most probable that their measurements are affected by some contribution from them.

Electrochemical reactions and adsorption of sulphurous species on electrode surfaces have been studied for a long time and have been a matter of concern [8, 9] Despite that, those effects are not completely clarified and, thus, more studies have been done and need to be done [10].

Actually, an upsurge of interest in their study appeared in recent years, due to increasing concerns on allergic reactions caused by sulfites added in food, though the actual cause of allergic reactions is a matter of controversy [11-13]. On the other hand, polyphenoloxidases are inhibited by sulfite [14-16].

The present work was, thus, part of the continuing efforts to get a better understanding of the interference of sulphurous species with polyphenol measurement. Our studies involved electrode surface reactions either in a fixed potential and amperometric detection mode or cyclic voltammetry, free or immobilised laccase inhibition and Folin-Ciocalteu measurements. The experiments were done in tartarate buffer at pH 3.5. At this pH, sodium metabisulfite will be mainly in the form of HSO_3^- , since $Na_2S_2O_5 + H_2O \rightleftharpoons 2NaHSO_3$ and

$SO_2 + H_2O \Leftrightarrow H_2SO_3 \underset{}{\overset{pK_1 \approx 2}{\rightleftharpoons}} H^+ + HSO_3^- \quad \underset{}{\overset{pK_2 \approx 7}{\rightleftharpoons}} 2H^+ + SO_3^{2-}$

2. EXPERIMENTAL

2.1. Reagents and solutions

Trametes Versicolor laccase (E.C.1.10.3.2, 22.4 U mg^{-1}) was from Fluka (Steinheim, Germany). Trans - 3,4- dihydroxycinnamic acid (caffeic acid), Folin-Ciocalteu's phenol reagent (FC reagent), disodium hydrogen phosphate (anhydrous) (Na_2HPO_4) and potassium dihydrogen phosphate (anhydrous) (KH_2PO_4), with purity superior to 99.9 % and 99.7 % respectively were obtained from Sigma–Aldrich (St. Louis, MO) [http://www.sigmaaldrich.com/sigma-aldrich/home.html]. L (+)-ascorbic acid, tartaric acid, NaCl with purity greater than 99.8 % and KOH were obtained from Riedel-deHaën (Seelze, Germany). Sodium metabisulfite, KCl purity superior to 99.5 % were from Merck (Darmstadt, Germany). Absolute ethanol, density 1.790 kg L^{-1} and sodium carbonate were obtained from Panreac Quimica AS (Barcelona, Spain). Polyethersulfone membranes (Ultrabind US450 0.45 µm) were from Gelman. Standard Caffeic acid solutions were prepared daily in tartarate buffer pH 3.5 at a concentration of 0.5 mM (100 mg L^{-1}).

The wine model solutions were prepared in (12% (v/v)) aqueous ethanolic 0.033 M tartarate buffer solution pH 3.5. They contained fixed caffeic acid and ascorbic acid concentrations (25 and 20 mg L^{-1}, respectively) and variable sodium metabisulfite (5 to 100mg L^{-1}). They were prepared daily. The solutions used for the enzyme immobilisation were 0.299 U μL^{-1} laccase (22.4 U mg $^{-1}$) was prepared in acetate buffer of pH 4.5. All aqueous solutions were prepared using water purified with a Milli-Q A10 system (Millipore).

2.2. Equipment and methods

Amperometric determination was made using a 4208 electrode base sensor, Pt–Ag, AgCl from Universal Sensors (New Orleans, LA). The electrode base system consisted of platinum wires 0.3 mm diameter and a silver, silver chloride helix which were contained in an electrode plastic cap closed on one end by an internal dialysis membrane. An amperometric biosensor detector (ABD) from Universal Sensors (New Orleans, USA) was used to apply the required potential and measure the consequent current obtained. The transients of current versus time were recorded on a Pharmacia Biotech recorder REC102.

Voltammetric measurements were carried out with a VoltaLab PGZ 100 from Radiometer Analytical connected to a PC software VoltaMaster 4 version 3.3. Two compartment cell with a Luggin capillary and three electrodes were used: the reference was a saturated calomel electrode (SCE) from Radiometer Analytical, the working electrode was a homemade Platinum disc having 5 mm diameter and the secondary electrode was a Platinum foil.

Spectrophotometric measurements were performed on a UV-Vis double beam Shimadzu UV-1603 spectrometer (Shimadzu, Kyoto, Japan) connected to a PC (UVPC ® Personal Spectroscopy Software).Operating conditions were set at 25 °C.

3. RESULTS AND DISCUSSION

3.1. Response of the sensor and the laccase-based biosensor to metabisulfite containing solutions

In order to differentiate the various contributions of metabisulfite to the final result obtained with the biosensor, different steps were taken.First of all the possibility of sulphurous compounds redox interactions at the electrode surface was checked. The electrode base system was dipped in the tartarate buffer, a potential of 100 mV vs Ag/AgCl was applied and the base current was allowed to reach a steady value, under magnetic stirring. After stabilization of the base current, injection of 200 µL of sodium metabisulfite (ranging from 5 to 40 mg L^{-1}) into the tartarate buffer solution was done. It was observed that the electrode base system responded to metabisulfite, a positive current being obtained. (It must be stressed that previous studies on caffeic acid did not yield any current, at the same applied potential, that is 100 mV vs Ag/AgCl, under similar circumstances).

In a second step, an enzyme containing membrane was put onto the base system and the current response to metabisulfite solutions, of the biosensor thus formed, had the same value as when there was no enzyme, evidencing that the enzyme was not actuating on the metabisulfite. The biosensor was prepared by direct immobilisation of 30 µL of laccase solution (8.96 U) on derivatized polyethersulfone membrane discs of 18 mm diameter. The optimization of the conditions for the biosensor had been previously done [17, 18]. The membranes with the immobilised enzyme were applied to a US electrode system base, above described. The external membrane containing the immobilised enzyme was juxtaposed to the internal membrane. Both were held tightly to the electrode plastic cap by an O-ring.

When a mixed solution of caffeic acid and metabisulfite was used, the response at the biosensor, that is, the system containing the enzyme, decreased with respect to the response to a pure solution of caffeic acid at the same concentration.

A 25 mg L^{-1} ascorbic acid solution did not give any response either with the sensor with only the internal membrane on, or the biosensor with the laccase membrane.

Experiments were, then, performed with mixed solutions containing fixed concentrations of ascorbic acid and caffeic acid and variable concentrations of metabisulfite. It was observed that there was an increase of the response of the sensor and biosensor with increasing concentration of metabisulfite. Moreover, when the current due to metabisulfite oxidation at the base sensor was subtracted from the current obtained with the biosensor, a constant value was obtained, that is an average value of -0.37 nA, with a standard deviation of 0.016 nA, which corresponded to the value obtained for the biosensor response to caffeic acid.

So, the sensor (with just the internal membrane on) responded to metabisulfites, the response being linear with the metabisulfite concentration. Subtracting the current caused by the metabisulfite reaction at the electrode from the current obtained when the biosensor was responding to the mixed solution, one obtained values for the current which were correspondent to the values obtained when the caffeic acid solution was pure.

After the assay with the mixed caffeic acid and metabisulfite solution, the biosensor response to caffeic acid was the same as before, which meant that the possible inhibition of the enzyme caused by the metabisulfite was reversible.

3.2. Measurement of the metabisulfite interference on determination of phenolic compounds by Folin–Ciocalteu method

The total phenolic content of wine model solution with fixed caffeic acid and ascorbic acid concentrations (25 and 20 mg L^{-1}, respectively) containing variable metabisulfite concentration (ranging from 2.5 to 100 mg L^{-1}) was determined according to the Folin-Ciocalteu (FC) colorimetric assay, with the micro scale protocol [19], which is based on the method reported in [20]. The total amount of polyphenols was determined by comparison with a standard solution of

caffeic acid [21]. The reaction took place directly in an eppendorf tube. For this purpose, 20 µL of wine model solution was pipetted into an eppendorf and 1.58 mL water was added, followed by 100 µL of the FC reagent. The mixture was allowed to stand for 1 min, and then 300 µL of the sodium carbonate solution (20 % (w/v)) were added. The reaction mixture was kept in the dark for 2 h. The absorbance was measured at 765 nm against the blank:

Absorbance = $0.001 Concn_{caffeic\ acid}$ (mg L^{-1}) – 0.0004; R^2 = 0.9986

The "total phenolic content" in the wine model solutions, was determined as Caffeic Acid Equivalents (CAE) – mg L^{-1}, by using an equation that was obtained from the standard caffeic acid plot. Results were means of three assays expressed as Caffeic Acid Equivalents (CAE) – mg L^{-1} of wine model solution. They are presented on figure 1.

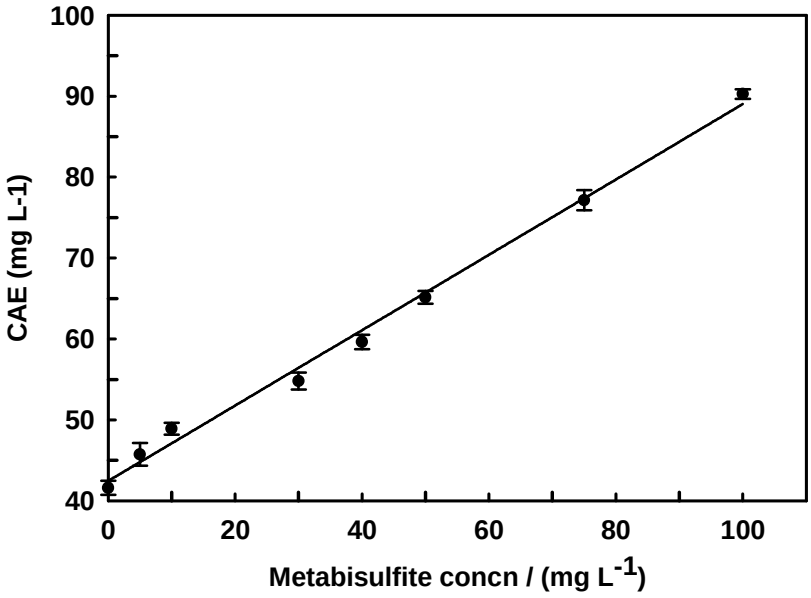

Figure 1. Caffeic acid equivalent of solutions containing a fixed amount of caffeic acid concentration and added metabisulfite at various concentrations as function of the latter referred concentrations.

3.3. Studies of the influence of metabisulfite on the activity of the enzyme laccase

3.3.1. Free laccase

In order to study the influence of metabisulfite on the oxidation of caffeic acid catalysed by laccase, addition of different amounts of metabisulfite to fixed caffeic acid concentration solution was done. The action of laccase was followed measuring the absorbance of an enzyme containing solution, at 321 nm, for 120 s. The reaction mixture consisted of 980 µL of wine model solution containing 0.083 mM caffeic acid (substrate), the inhibitor compound (metabisulfite) (ranging from 0.5 to 40 mg L^{-1}) and 20 µL of laccase solution (0.05 U).The reaction was run at 25°C and started by the addition of substrate solution to the enzyme solution. The caffeic acid oxidation rate was determined spectrophotometrically at 321 nm by **monitoring** substrate **consumption.** A control reaction was carried out using wine model solution containing 0.083 mM caffeic acid (substrate) without inhibitor. The activity was obtained from the slope of the first 10 seconds of the graph. The resultant graph of vi vs metabisulfite concentration (mg L^{-1}) is shown on figure 2.

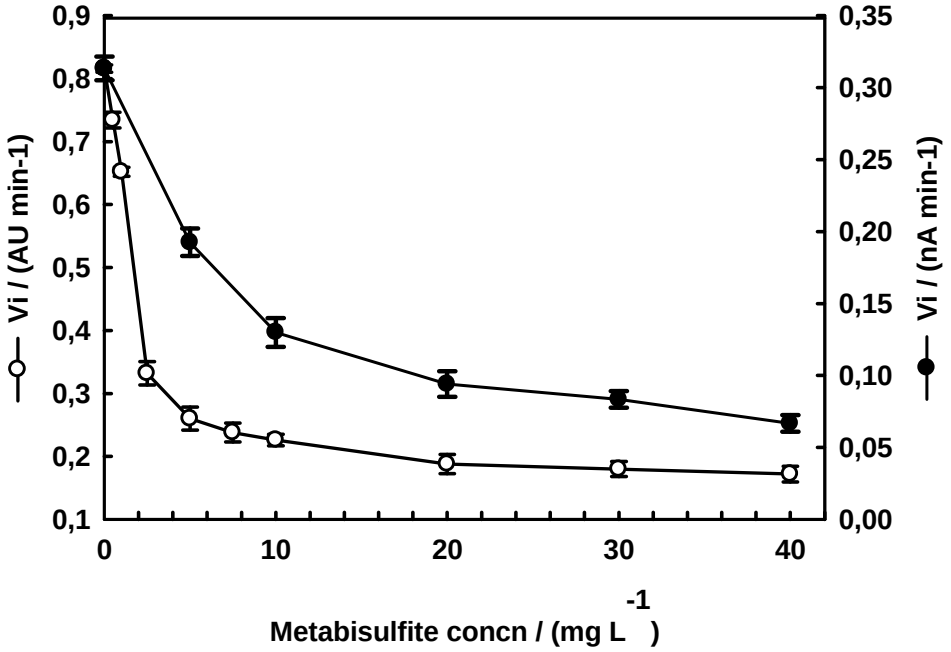

Figure 2. Metabisulfite inhibition of the activity of free (o) and immobilized (•) laccase on the oxidation of caffeic acid.

It was, thus, evidenced that metabisulfite inhibited the activity of free laccase with respect to caffeic acid oxidation. The inhibition percentage was calculated by means of the formula:

$$I = 100 - \left(\frac{V_{iSample}}{V_{iControl}} \right) \times 100 \qquad (1)$$

where, I: inhibition (%), Vi sample: initial oxidation rate (AU min^{-1}) , and Vi control: initial oxidation rate in control test (AU min^{-1}). Tests were carried out in triplicate and a blank with tartarate buffer instead of enzyme solution was done.

The half-maximal inhibitory concentration (IC_{50}) value was obtained plotting the inhibition percentage against metabisulfite solution concentrations. IC_{50} was equal to 2.16 ± 0.09 mg L^{-1}.

3.3.2. Immobilised enzyme

The presence of metabisulfite and the corresponding influence on the laccase action on caffeic acid, when used in the biosensor, was also studied. Thus, wine model solution containing caffeic acid (substrate) and the inhibitor, metabisulfite (5 to 40 mg L^{-1}) was measured using the same biosensor methodology as above described. The transients of current versus time were recorded and the respective initial rate was determined as the slope of the initial current (nA) versus time (min).

It was observed that there was a decrease in the speed of the biosensor to reach a steady response. Figure 2 contains the corresponding graph of the vi (nA min^{-1}) vs metabisulfite concentration.

Although the response of the biosensor was inhibited by metabisulfite, the enzyme was still active up to a metabisulfite concentration of 40 mg L^{-1} and catalyzed the oxidation of caffeic acid, though the time for the reduction current to reach a steady value tended to be longer.
Inhibition (%) was calculated in the following way:

$$I = 100 - \left(\frac{V_{iSample}}{V_{iControl}} \right) \times 100 \qquad (2)$$

where, I: inhibition (%), Vi sample : initial oxidation rate (nA min^{-1}), and Vi control: initial oxidation rate in control test (without inhibitor) (nA min^{-1}). Tests were carried out in triplicate and a blank with a polyethersulfone membrane without immobilised enzyme was done.

Metabisulfite concentration providing 50 % inhibition (IC_{50}) was obtained plotting the inhibition percentage against metabisulfite solution concentrations. The IC_{50} was equal to 8.18 ± 0.14 mg L^{-1}. Thus, laccase immobilised on polyethersulfone membranes is less inhibited by metabisulfite than free laccase.

3.4. Cyclic voltammetry analysis

The influence of metabisulfite on the platinum electrode was also analysed by cyclic voltammetry. Solutions of sodium metabisulfite (10 to 100 mg L^{-1}) in tartarate buffer pH 3.5 and mixed solutions containing ascorbic acid (20 mg L^{-1}) and caffeic acid (25 mg L^{-1}), besides sodium metabisulfite were analysed. Platinum working electrode was polished with 0.05 µm alumina. Platinum auxiliary electrode was cleaned with concentrated HNO_3 and thoroughly washed with distilled water. Voltammograms were run from -800 to 900 mV vs SCE at a scan rate of 100 mV s^{-1}. The cyclic voltammograms obtained are shown on figures 3 and 4.

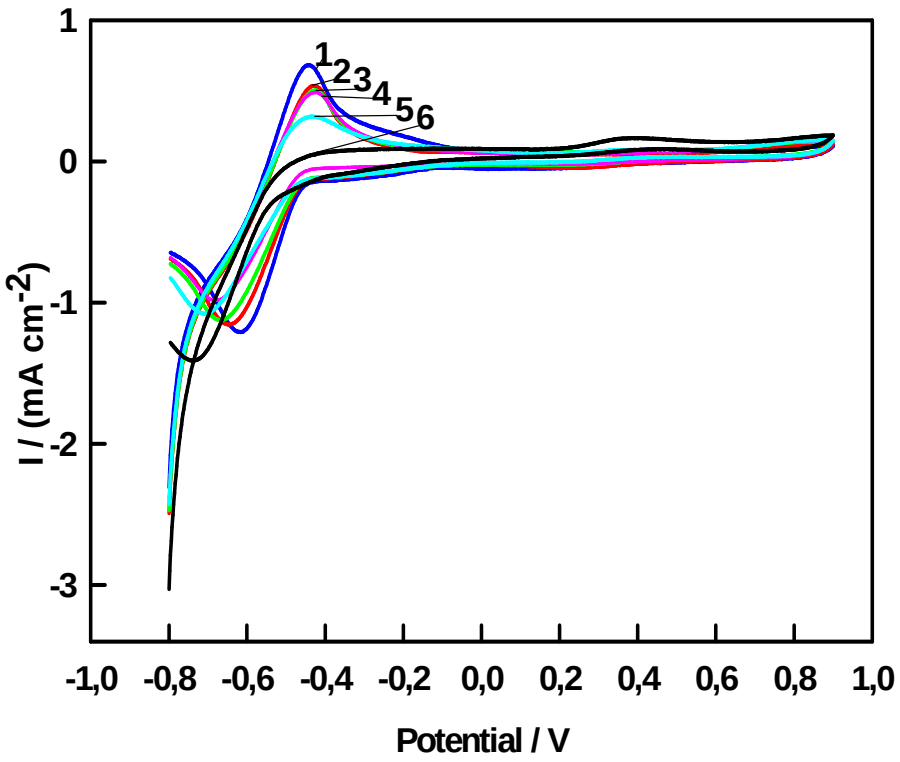

Figure 3. Cyclic voltammograms of sodium metabisulfite at: curve 1 – 10; curve 2 – 20; curve 3 – 30; curve 4 – 40; curve 5 – 50 and curve 6 – 100 mg L^{-1} in tartarate buffer pH 3.5, from -800 to 900 mV, at scan rate 100 mV s^{-1}. WE platinum disc; reference electrode: SCE.

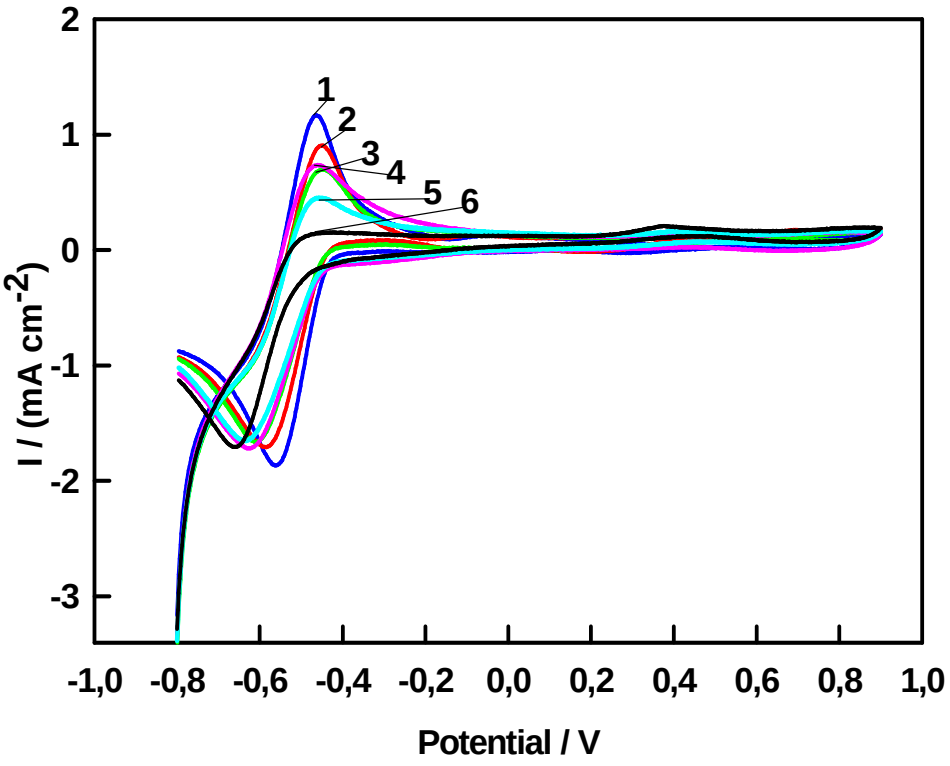

Figure 4. Cyclic voltammograms of sodium metabisulfite at: curve 1 – 10; curve 2 – 20; curve 3 – 30; curve 4 – 40; curve 5 – 50 and curve 6 – 100 mg L^{-1} with added ascorbic acid at mg L^{-1} and caffeic acid at 25 mg L^{-1} in tartarate buffer pH 3.5 , from -800 to 900 mV, at scan rate 100 mV s^{-1}. WE platinum disc; reference electrode: SCE.

A well defined anodic peak was observed in the region -0.6 V to -0.3 V vs SCE, in the anodic potential direction. There was a corresponding cathodic peak in the region -0.5 to -0.8 when the scan was reverted in the cathodic direction. Those peaks could be ascribed to the hydrogen adsorption/desorption reactions [22, 23].

The lowering of the intensity of the current due to the main adsorption reduction/oxidation states of hydrogen and its eventual disappearance has been explained by adsorption of sulfite species before their oxidation. Sulfite and/or intermediate products are strongly adsorbed on the catalytic sites with subsequent partial replacement of hydrogen atoms**"** [24].

It was observed that the same pattern of adsorption reduction/oxidation states of hydrogen was obtained on pure solutions of potassium metabisulfite and mixed solutions containing also ascorbic acid and caffeic acid. The intensity of the anodic peak currents varied linearly with the concentration of metabisulfite. The corresponding equations were: I_{pa} (mA cm^{-2}) = - 0.0061 C + 0.8982, R^2 = 0.9782 for solutions of metabisulfite, at concentrations x mg L^{-1}, in tartarate buffer pH 3.5 and I_{pa} (mA cm^{-2}) = - 0.0094 C + 1.3905, R^2 = 0.9325 for mixed solutions containing metabisulfite, at concentrations x mg L^{-1} plus 20 mg L^{-1} ascorbic acid and 25 mg L^{-1}, in tartarate buffer pH 3.5. It was, thus, confirmed that the main contribution to reducing the hydrogen adsorption and desorption reactions was from metabisulfite, although ascorbic acid and caffeic acid also contributed, as evidenced by the increase of the slope.

4. CONCLUSIONS

Sodium metabisulfite was found to influence both measurements taken with laccase-based biosensors and Folin-Ciocalteu spectrophotometric method. So, following the statement of those who said that an “index” could be accepted as an approach to know the “total polyphenolic” content, we support the recommendation that results obtained in wine with laccase-based biosensors and Folin-Ciocalteu spectrophotometric method should be reported as “caffeic acid equivalent”.

ACKNOWLEDGEMENTS

We thank Fundação para a Ciência e a Tecnologia for the grant: SFRH/BD/34511/2006 and Financial support from Faculdade de Ciências da Universidade de Lisboa. Helpful discussions with Prof. Inês Fonseca are gratefully aknowledged.

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ARTICLE III

Gallic acid interference on polyphenolic amperometric biosensing using *Trametes versicolor* laccase

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Journal of Molecular Catalysis B: Enzymatic, *submitted*

ABSTRACT

The present work reports the gallic acid (GA) interference on polyphenolic amperometric biosensing using *Trametes versicolor* laccase (TvLac). GA' inhibitory effect on TvLac activity was investigated on the oxidation of caffeic acid (CA) by free Tvlac and its immobilised form on modified polyethersulfone membrane (PES/TvLac), using spectrophotometric and amperometric biosensor detection methods. The results have indicated that GA presents inhibitory behaviour on TvLac activity in a concentration-dependent manner. The GA concentration leading to 50% activity lost, $IC_{50,}$ was determined to be 19.15 ± 0.11 µM and 5.11 ± 0.19 µM for free and immobilised enzyme, respectively. The results have also shown that GA exhibited a competitive and a mixed inhibition types on the TvLac activity for spectrophotometric and amperometric biosensor methods, respectively. Further GA' and CA' cyclic voltammetry studies have demonstrated that GA' oxidation products interfered with CA' redox reaction products. In fact, a decrease of the reduction current was observed at cyclic voltammograms of CA, when mixed with GA. Therefore, the GA' interference on polyphenolic amperometric biosensing is the result of the combination of two factors: on one hand, we have the inhibitory enzymatic effect, and on the other, the reaction of GA' oxidation products with the o-quinones obtained by the enzymatic oxidation of CA. Both gave rise to the amperometric signal decreasing effect.

Keywords: Gallic acid; polyphenol; *Trametes versicolor*; inhibitory activity; amperometric biosensor.

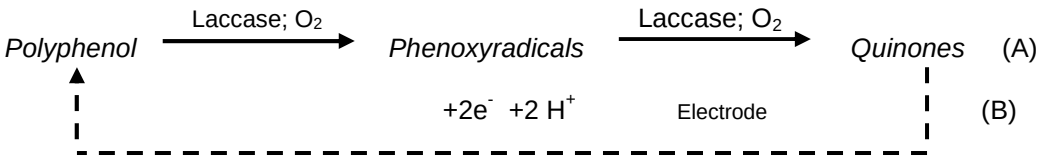
1. INTRODUCTION

Trametes versicolor belongs to the white-rot basidiomycetes fungi and produces an extracellular enzyme named laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) (TvLac) [1]. TvLac is a blue multicopper oxidase enzyme which uses molecular oxygen to oxidize phenolic and non-phenolic compounds [2, 3]. In a typical laccase reaction (Scheme 1A) the phenolic substrate undergoes a one-electron oxidation generating a free radical (phenoxyradical). This active oxygen species, typically unstable, can be converted into a quinone in a second oxidation step [4].

Laccases are attractive for many biotechnological applications such as biosensors for analysis of phenolic compounds in blood, wine, tea, fruit juice, oil and medicinal herbs extracts [5]. In this context laccases from various sources have been successfully immobilised on various supports for use as biological recognition element of electrochemical biosensors [[1] and references therein]. In the laccase-based amperometric biosensors, the quinones produced by the second stage of enzymatic oxidation can be electrochemically reduced to the initial phenolic compound, at electrode surfaces, at an appropriate potential (Scheme 1B). The measured current is proportional to the concentration of phenolic compounds.

Son et al [6] have reported that certain phenolic acids inhibit tyrosinase (a polyphenol oxidase) activity by binding to the active site of the enzyme. These authors reported that gallic acid (GA; 3,4,5-trihydroxybenzoic acid) showed inhibitory activity on tyrosinase similar to ascorbic acid. Thus, GA has been described as an inhibitor of tyrosinase activity, and yet, GA has also been referred as a tyrosinase substrate, being oxidised by the enzyme [7, 8]. It is known that TvLac belongs to the well studied laccases [9], however to our knowledge, there are no studies in the literature regarding TvLac inhibition by GA. Therefore, the purpose of the present work was to investigate the possibility of GA interference on polyphenolic amperometric biosensing using TvLac. In fact, GA is found in most foods and plants, being one of the most important simple polyphenolic units of tannins. Thus, GA' inhibitory effect on TvLac activity was investigated on the oxidation of caffeic acid (CA) by free Tvlac and its immobilised form on modified

polyethersulfone membrane (PES/TvLac), using comparative spectrophotometric and amperometric biosensor detection methods. Subsequently, the electrochemical behavior of CA and GA was also studied by cyclic voltammetry, in order to verify a possible interfering effect on the amperometric sensor system.



Scheme 1 Reaction of polyphenol substrate oxidation catalysed by laccase.

2. MATERIALS AND METHODS

2.1. Reagents and solutions

Trametes versicolor laccase (E.C.1.10.3.2, 30.6 U mg $^{-1}$) was purchased from Fluka (Steinheim, Germany). Gallic acid, caffeic acid, catechin, chlorogenic acid, rutin, rosmarinic acid were obtained from Sigma–Aldrich (St. Louis, MO) [http://www.sigmaaldrich.com]. L (+) tartaric acid was obtained from Riedel-deHaën (Seelze, Germany). Absolute ethanol, density 1.790 kg L^{-1} was obtained from Panreac Quimica AS (Barcelona, Spain) [http://www.panreac.es/]. Polyethersulfone membranes (PES) (Ultrabind US450 0.45 µm) were from Gelman [http://www.pall.com]. All other chemical reagents used were of analytical grade. All aqueous solutions were prepared using water purified with a Milli-Q A10 system (Millipore). Stock solutions of phenolic compounds (10 mg mL^{-1}) were prepared in ethanol. The assay solutions were prepared by diluting the stock solutions in 0.033 M tartarate buffer pH 3.5.

2.2. TvLac immobilisation

The concentration of immobilised TvLac was fixed in all the experiments. The enzyme, laccase (TvLac) at 9.8 mg mL^{-1} solution, was prepared in acetate buffer of pH 4.5. The covalent immobilisation on modified PES membranes (PES/Tvlac) was performed according to the method we previously reported [10-12].

2.3. Amperometric detection of phenolic compounds

All the studies in this paper were carried out at least in triplicate and the operating conditions were set at 25℃. In a previous paper [13], the dev elopment of a TvLac - based biosensor was presented. All measurements were performed using a 4208 electrode base sensor, Pt–Ag, AgCl from Universal Sensors (New Orleans, LA). The electrode base system consisted of platinum wires 0.3 mm diameter and silver, silver chloride helix which were contained in an electrode plastic cap closed on one end by an internal dialysis membrane. An amperometric biosensor detector (ABD) from Universal Sensors (New Orleans, USA) was used to apply the required potential and measure the consequent current obtained. The transients of current versus time were recorded on a Pharmacia Biotech recorder REC102. Amperometric measurements were done as follows: the bioelectrode was prepared by attaching the enzyme-modified membrane (PES/Tvlac) to the electrode base system, with an o-ring. The bioelectrode was immersed in 5 mL of 0.033 M tartarate buffer solution pH 3.5, the potential was applied and, when the base current attained a stable value, 50 µL of substrate (phenolic) solution was directly added under continuous magnetic stirring at 300 rpm. The experiments were carried out at a constant potential (100 mV versus Ag/AgCl) and the current response corresponding to the reduction of enzymatically produced o-quinone was recorded as function of time.

2.4. Cyclic voltammetry

Cyclic voltammetry was performed using VoltaLab PGZ 100 from Radiometer Analytical connected to a PC software VoltaMaster 4 version 3.3. Experiments were carried out in a three electrode cell with 10 mL of tartarate buffer pH 3.5. The auxiliary electrode was a platinum wire, the reference was a saturated calomel electrode (SCE) from Schott Instruments and, the working electrode was a 3-mm glassy carbon disk (GCE) (BAS010420) (BAS Inc., Tokyo, Japan) which was cleaned by polishing in 0.05µm alumina powder for 30 s and rinsed with deionised water in an ultrasonic bath for 3 min. In order to have electrochemical reproducible results the GCE was cleaned after each voltammogram experiment.

2.5. Spectroscopic and TvLac activity assays

All spectroscopic and spectrophotometric assays (free TvLac activity assays) were carried out on a UV-Vis double beam Shimadzu UV-1603 spectrometer (Shimadzu, Kyoto, Japan) connected to a PC (UVPC® Personal Spectroscopy Software). UV-visible absorption-spectroscopy studies were performed in 1cm path cuvette and in 0.033 M tartarate buffer pH 3.5 containing 50 µM CA or 50 µM GA or mixture of both phenolics and TvLac solution (0.10 U). All enzymatic reactions were performed in 0.033 M tartarate buffer pH 3.5. The final volume of all the reactions mixtures was 1.2 mL in a 3 mL, 1cm width, UV-Vis cuvette. The rate of the enzymatic oxidation reaction of CA and GA were determined spectrophotometrically by monitoring substrate consumption: CA (λ_{max} = 321 nm; $\varepsilon_{321\ nm}$ = 13700 $M^{-1}cm^{-1}$, pH 3.5) and GA (λ_{max} = 270 nm; $\varepsilon_{270\ nm}$ = 9100 $M^{-1}cm^{-1}$, pH 3.5), respectively for two minutes. The activity assay mixture consisted of 100 µL enzyme solution (0.033 mg mL^{-1}), 100 µL of tartarate buffer pH 3.5 and 1000 µL of substrate solution. CA' and GA' concentrations ranged from 0.02 to 0.2 mM and 0.05 to 0.5 mM, respectively. The activity was obtained from the slope of the first 10 seconds of the graph and was calculated in international unit (U) which is the amount of Tvlac that oxidises one µmol of substrate per min at 25 ºC. The Michaelis-Menten constants for the substrates were obtained by regression of the measured values.

2.6. Inhibition studies

The inhibition kinetic assays were performed to determine the type of inhibition on free and immobilised Tvlac (PES/TvLac) by GA. A typical enzyme inhibition assay was designed to determine the nature of the inhibition process. For this purpose, a different set of assays was carried out at constant concentration of GA (5 to 10 µM) and varying the concentration of CA (0.02-0.2 mM). The activity assay was performed as described above, replacing 100 µL of tartarate buffer pH 3.5 by 100 µL of inhibitor solution and, monitoring the associate decrease in absorbance at 321 nm. This decrease is proportional to the rate of CA consumption. The assays for immobilised Tvlac (PES/TvLac) were performed amperometrically, as described above, and initial velocities (nA min^{-1}) were determined at CA concentration in the absence of gallic, as well as with added GA at concentrations ranging from 5 to 10 µM. To assess the GA concentration needed to reduce TvLac activity by 50% (IC_{50}), the experiments were carried out at constant concentration of CA (0.015 mM) and varying the concentration of GA (1 to 150 µM) and (1 to 30 µM) for free and immobilised TvLac, respectively. A control reaction was carried out using 0.015 mM CA without inhibitor. The inhibition percentage was calculated by means of the formula: $I = 100 - \left(\frac{V_{i\ sample}}{V_{i\ control}} \right) \times 100$ where, I: inhibition (%), $V_{i_{sample}}$: initial oxidation rate of the sample and $V_{i_{control}}$: initial oxidation rate in control test. The IC_{50} values were obtained from the plot of the logarithm of GA concentrations against the percent inhibition of the enzyme activities.

3. RESULTS AND DISCUSSION

3.1. GA' negative synergetic effect on the biosensing reduction current

The influence of phenolics interactions on the biosensing reduction current was studied for six compounds: caffeic acid, catechin, chlorogenic acid, rosmarinic acid, rutin and gallic acid. Each phenolic compound was at a concentration of 5 µM, except GA that was at 50 µM.

Firstly, the possibility of phenolic compounds redox interactions at the electrode surface was investigated. It was observed that the electrode base system did not respond to those compounds at the applied potential studied. Afterwards, each individual compound and successive combined mixed solution was analysed using the amperometric biosensing system for phenolic detection. That assay consisted of measuring the current signal variation (ΔI (nA)) corresponding to the reduction of enzymatically produced o-quinones at the electrode, as showed on scheme 1. Table 1 shows the effect of phenolic compounds on the biosensor response in terms of reduction current (nA). The above results have indicated that phenolic mixtures presented two different effects on the biosensing response. Four mixtures (M_1, M_2, M_3 and M_4) showed an additive reduction current behaviour resulting on the summation of individual reduction currents.

However, the addition of mixture M_5, containing GA, led to a decrease on biosensing detection. Thus, GA has demonstrated a negative synergetic effect on the biosensing reduction current. It must be stressed that the biosensor did not respond to a 5 µM GA solution on itself. Possibly, GA' negative synergetic behaviour may be attributed to chemical interactions among phenolic molecules, a lower TvLac specificity towards GA and/or an inhibitory effect on TvLac activity.

Table 1. Amperometric detection of phenolic compounds.

Phenolic compounds[a]	Biosensor response, ΔI (nA)[b]
Individual	
1. Caffeic acid, 5 µM	- 0.42 ± 0.02
2. Catechin, 5 µM	- 0.28 ± 0.01
3. Chlorogenic acid, 5 µM	- 0.29 ± 0.01
4. Rosmarinic acid, 5 µM	- 0.21 ± 0.02
5. Rutin, 5 µM	- 0.09 ± 0.01
6. Gallic acid, 50 µM	- 0.04 ± 0.01
Mixture	
M_1 = 1+ 2	- 0.71 ± 0.01
M_2 = M_1+ 3	- 1.00 ± 0.03
M_3 = M_2+ 4	- 1.20 ± 0.03
M_4 = M_3+5	- 1.28 ± 0.02
M_5 = M_4+ 6	- 0.05 ± 0.02
M_0 = 1+ 6	- 0.02 ± 0.01

[a] Phenolic solutions in 0.033 M tartarate buffer, pH 3.5.

[b] Data are presented as means ± standard deviation of reduction current (n=3).

In order to investigate these possibilities, GA inhibitory enzymatic activity studies were performed with spectrophotometric UV-Vis detection for free TvLac. Parallel studies were done for immobilised TvLac on modified PES membranes (PES/TvLac) with amperometric detection – that was the biosensor case, and following the redox response by cyclic voltammetry.

CA and GA were used in the subsequent experiments. First of all, the reason of this choice was due to the fact that CA (3,4-dihydroxycinnamic acid) and GA (3,4,5-trihydroxybenzoic acid) are structurally similar, both have a catechol skeleton, though GA has an additional hydroxyl group as shown on Table 2. Additionally, CA was the phenolic compound studied in this work that presented the highest biosensing reduction current as shown on Table 1. Moreover, the mixture of CA (5 µM) and GA (50 µM) (M_0) exhibited a strong negative synergism effect as shown on Table 1.

3.2. UV-visible absorption-spectroscopy studies

UV-visible absorption-spectroscopy studies were carried out in order to observe the effects of the chemical interaction among phenolic molecules and its corresponding enzymatically oxidised products.

Preliminary studies were done following the absorption spectra from control solutions containing only CA, GA and a mixture of both phenolic compounds, for 30 minutes. Figure 1A shows the corresponding overlayed UV-Vis spectra at pH 3.5. The absorption spectra of the mixed solution showed no variations with respect to the summation of the spectra of the separate compounds, thus suggesting that no interactions and/or autoxidation occurred in a 30 minute period for individual and combined phenolics. The results showed that the absorption spectra of CA and GA solutions had an absorbance maximum at 321 nm and 270 nm, respectively. These data are in accordance with those reported in the literature [14, 15].

Enzymatic oxidation of CA, GA and a mixture of both phenolic acids, by TvLac, was followed by UV-Vis absorption at pH 3.5, and the respective spectra are displayed on Figure 1B and 1C. Figure 1B shows that enzymatic oxidation has been characterised by the appearance of a new peak with the maximum at 405 nm and 385 nm for CA and GA respectively. Those news peaks presumably corresponded to the formed o-quinone absorption [7, 16]. Figure 1B shows that TvLac oxidised both compounds and also, apparently, seemed to have a higher action on CA, giving rise to the corresponding o-quinone, which exhibited a maximum absorbance at 405 nm. However, when CA was oxidised in the presence of GA, the maximum absorbance was shifted up to 450 nm, resulting in a large band with maximum absorbance around 430-450 nm as shown on Figure 1C. The enzymatic oxidation of the phenolic acids results in their corresponding o-quinones, which then undergo non-enzymatic reactions, generating polymeric phenolic compounds, since they are reactive oxidizing agents and electrophiles. It is often suggested that brown pigments may have been due to the oxidative polymerisation reactions induced by the o-quinone oxidation product [7, 17]. The spectroscopic studies evidenced that o-quinones resulting from phenolic acids, were rather unstable, which is a characteristic behaviour of many o-quinones. In fact, a decrease of the maximum of absorbance band around 430-450 nm, as function of time, was observed confirming the enzymatically oxidised end products disappearance, as shown on Figure 1C (iii, iv, v).

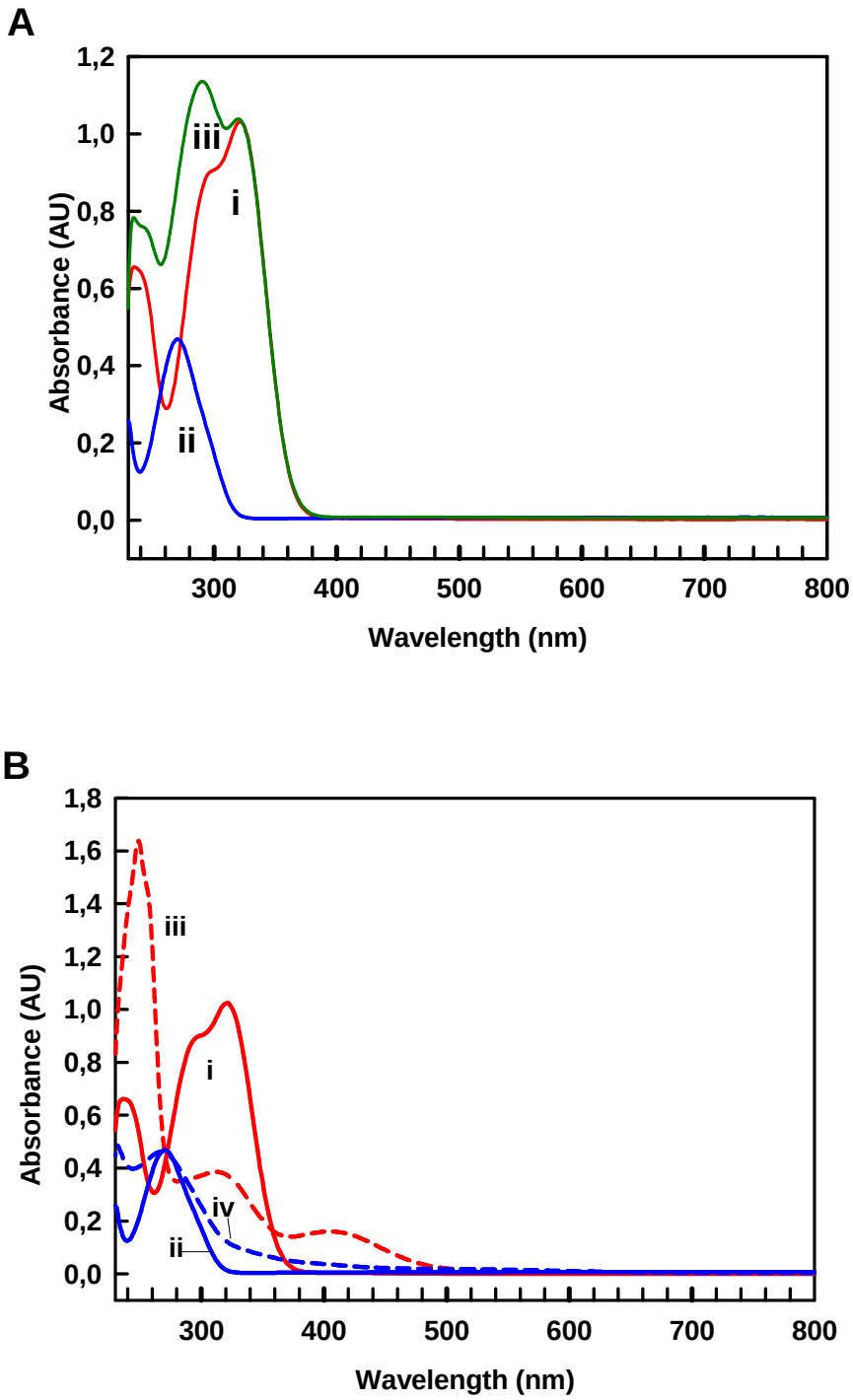


Figure 1. UV-Vis absorbance spectra at pH 3.5. Initial conditions: caffeic acid (50 µM); gallic acid (50 µM) and Tvlac (0.10 U). **A** - Caffeic acid (i), gallic acid (ii), and a mixture of both phenolic compounds (iii). **B** - Caffeic acid (i) and gallic acid (ii) oxidation by TvLac and respective enzymatically-oxidised end products: (iii) from (i); and (iv) from (ii), at 2 min of reaction.

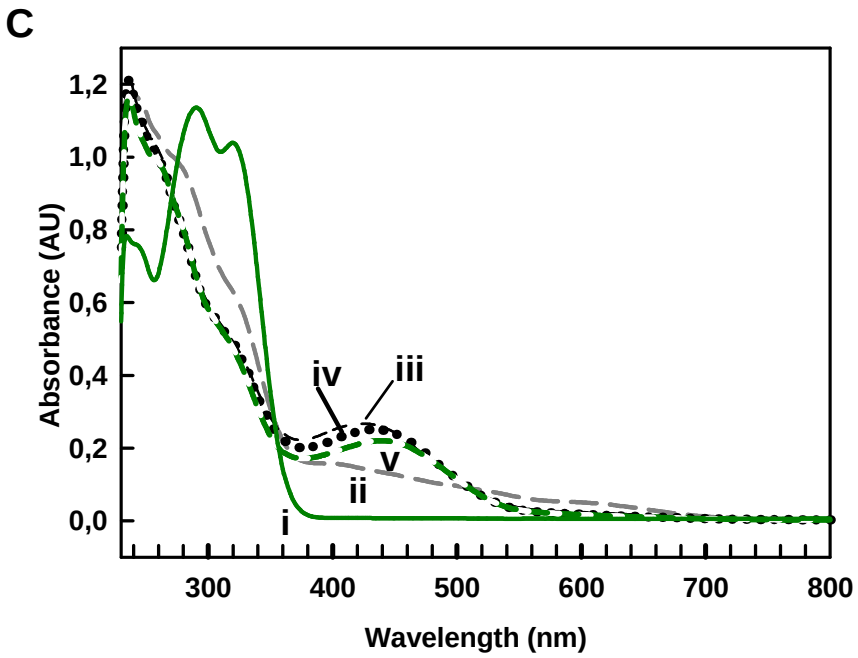


Figure 1. UV-Vis absorbance spectra at pH 3.5. Initial conditions: caffeic acid (50 µM); gallic acid (50 µM) and Tvlac (0.10 U). **C** - Mixture of caffeic acid and gallic acid oxidation by TvLac at five instants: (i) 0, (ii) 2, (iii) 4, (iv) 6, and (v) 8 min of enzymatic reaction.

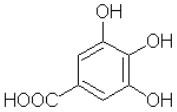
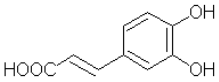
3.3. TvLac substrate specificity

Our spectroscopic studies showed, as expected, that GA and CA were oxidised by TvLac. The kinetic parameters of enzyme biocatalysis using GA and CA as substrates were studied to elucidate about TvLac substrate specificity.

Michaelis-Menten kinetics between the substrate concentration and the initial rate of the TvLac oxidation reaction has been found [18]. Table 2 shows the kinetic parameters obtained from a Lineweaver-Burk plot: the Michaelis-Menten constant (K_m) and the maximum reaction rate (V_{max}). We found that TvLac had an almost 5-fold higher K_m to CA (K_m = 0.11 ± 0.02 mM) than to GA (K_m = 0.53 ± 0.05 mM).

The catalytic efficiency expressed as the V_{max} / K_m ratio, was also used as an indication of the ability of TvLac to convert substrates into corresponding end products. TvLac catalytic efficiency for CA was greater (*ca.* 47 orders of magnitude) then for GA as shown on table 2. Some authors [6, 19, 20] have also reported that CA was more efficiently oxidised then GA by enzymes such as tyrosinase and laccase.

Table 2. Substrate specificity of TvLac.

Substrate	K_m[a] (mM)	V_{max}[a] (µmol mg^{-1} s^{-1})	V_{max} / K_m (mg^{-1} L^{-1} s^{-1})	Specificity[b] (%)
Gallic acid 	0.53 ± 0.05	8 ± 0.4	0.015 ± 0.062	2.12
Caffeic acid 	0.11 ± 0.02	78 ± 5	0.709 ± 0.016	100

[a] *K*m, Michaelis–Menten saturation constant; *V*max, maximum reaction rate of oxidation.
Data was obtained from Lineweaver-Burk plots where *x* intercept = -1/K_m, and *y* intercept = 1/V_{max}. The data are presented as means ± standard deviation (n=3).
[b] The catalysis efficiency was defined as ratio of V_{max} to K_m, the percentage of specificity was calculated using caffeic acid as reference.

3.4. TvLac activity inhibition by GA

The effect of GA on oxidative reaction of CA was studied on free and immobilised TvLac (PES/TvLac) using spectrophotometric and amperometric biosensor methods, respectively. The relationship between residual enzyme activity and the concentration of GA is shown on Figure 2A. The result show that GA inhibited TvLac activity and that inhibition was concentration dependent. In fact, the residual TvLac activity decreased as the concentration of GA increased.

In this study, the concentration of GA that decreased the rate of the enzymatic oxidation reaction of CA by 50% (IC_{50}) was obtained plotting the inhibition percentage against the logarithm of the concentration of GA solution as shown on Figure 2B.

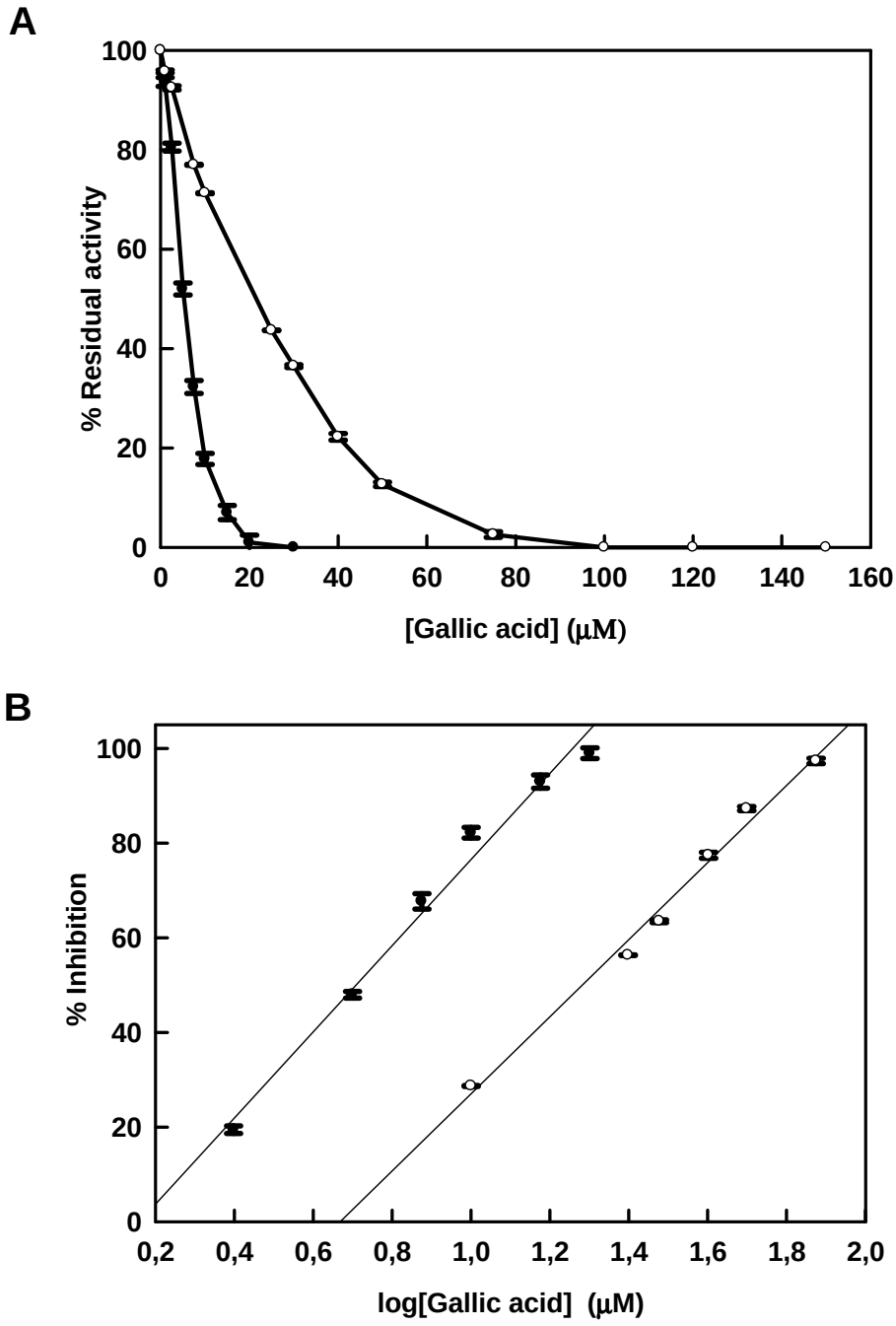


Figure 2. **A**-Effect of gallic acid on the activity of Tvlac. **B**-Inhibition curves for gallic acid. Caffeic acid (0.015 mM) was used as substrate; free TvLac (spectrophotometric method) (○) and immobilised (PES/TvLac) (amperometric biosensor method) (●).

The free TvLac assay was less sensitive towards GA inhibition (IC_{50} = 19.15 ± 0.11 µM) than the TvLac immobilised on modified PES membranes (IC_{50} = 5.11 ± 0.19 µM). These results indicated that GA inhibition was approximately four times more effective on immobilised enzyme.

Furthermore, the inhibition kinetics of GA on free and immobilised TvLac (PES/TvLac) were analysed by Lineweaver-Burk plots as shown on Figure 3. The four lines were obtained from the uninhibited enzyme activity on CA and from three different concentrations of GA (5, 7.5 and 10 µM). For free TvLac the lines intersect on the vertical axis as shown on Figure3A. This result indicated that GA exhibited a competitive inhibition for CA oxidation by TvLac. However, immobilised PES/TvLac exhibited a mixed inhibition, as revealed on Figure 3B. These findings indicated that PES/TvLac associated with amperometric biosensor method provided a more favourable microenvironment to GA inhibitory activity than free enzyme.

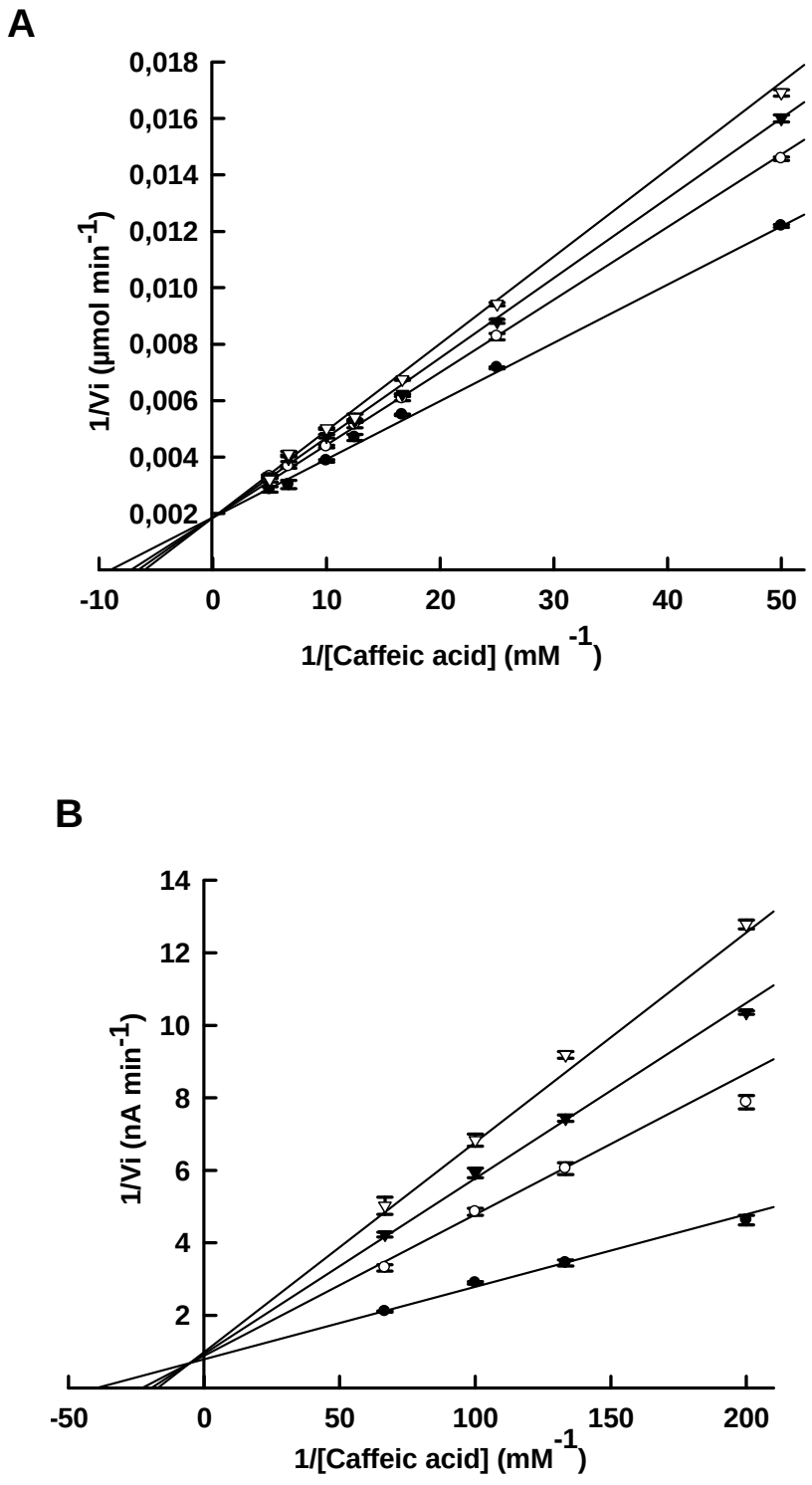


Figure 3. Lineweaver-Burk double reciprocal plots showing inhibition of TvLac by gallic acid: (●) control, (○) 5 µM, (▼) 7.5 µM and, (▽) 10 µM. Caffeic acid was used as substrate. A- free TvLac (spectrophotometric method); B- immobilised (PES/TvLac) (amperometric biosensor method).

3.5. Cyclic voltammetry studies

It is important to notice that polyphenols are redox cycled between enzymatic oxidation and electrochemical reduction giving a response at TvLac modified electrode. The responses should be proportional to the concentration of the phenolic compounds in the solution, if they are meant to have analytical application. In order to better understand the electrochemical behavior of GA and CA, some experiments were performed using cycling voltammetry at GCE, in the range of 200 to 600 mV (vs. SCE), at a scan rate of 100 mV s^{-1}. The pH value of the solutions was 3.5.

The cyclic voltammogram (CV) of 50 µM CA is shown on Figure 4A. The anodic peak potential (E_{pa}) was at 448 mV, which corresponds to the oxidation of the phenolic hydroxyl groups of CA, and the cathodic peak potential (E_{pc}) at 416 mV, which corresponds to the reduction of the formed o-quinone. The separation between the anodic and cathodic peak potential, $\Delta E_p = |E_{pc} - E_{pa}| = 32$ mV, and the ratio of the anodic peak current to the cathodic one, $I_{pc}/I_{pa} = 0.93$, confirmed that the electrochemical process of CA at a GCE was reversible under these conditions.

Curves a-f in Figure 4B show the cyclic voltammograms of 12.5; 25; 50; 125; 250 and 500 µM GA solutions, respectively. Cyclic voltammetric experiments showed a single oxidation peak at 470 mV, very similar in size and shape to that produced by CA [16, 21], and on the reverse scan, no reduction peak was observed, showing that GA was irreversibly oxidized at the GCE, suggesting an oxidation process coupled with a fast subsequent chemical reaction [21].

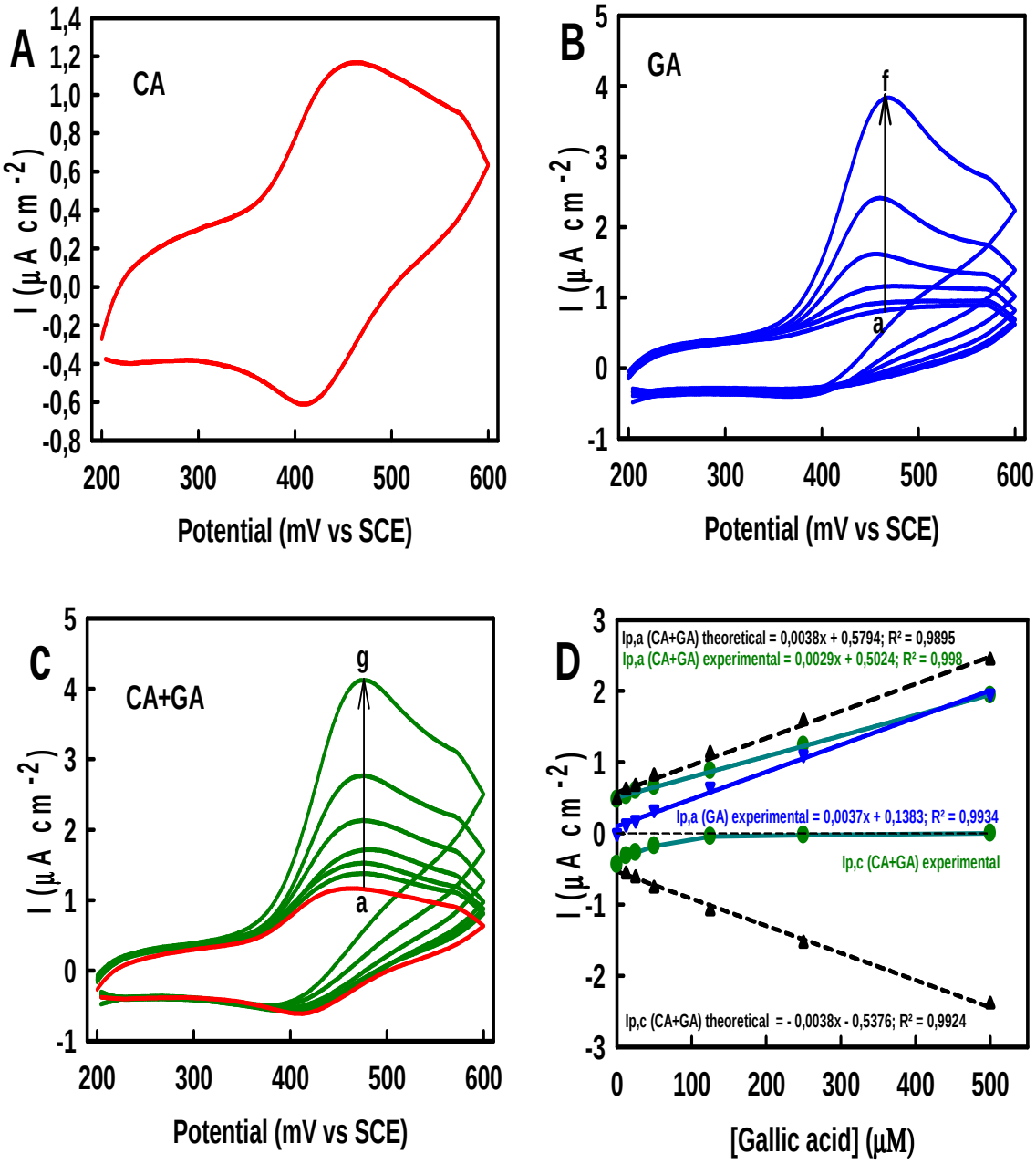


Figure 4. Cyclic voltammograms at pH 3.5 were obtained in the range of 200 to 600 mV at scan rate of 100 mV s^{-1}. WE, 3 mm GCE.

- **A-** Caffeic acid (50 µM);
- **B-** Gallic acid (12.5 to 500 µM) corresponding to a- f, respectively;
- **C-** Mixture of both phenolic compounds at fixed concentration of caffeic acid (50 µM) and varying the gallic acid concentration from 0 to 500 µM, corresponding to a-g, respectively;
- **D-** Current peak versus gallic acid concentration from 12.5 to 500 µM, and at fixed concentration of caffeic acid (50 µM) with respective gallic acid concentration from 0 to 500 µM.

The CV's showed successive enhancement of peak current on increasing GA concentration. The plot of peak current versus the respective concentration of GA was found to be linear in the range 12.5 to 500 µM, I_{pa} (GA) $_{experimental}$ = 0,0037 [GA] + 0,1383; R^2 = 0,9934 as shown on Figure 4D.

The oxidation of 50 µM CA to the corresponding o-quinone, in the presence of GA (0 to 500 µM) was also studied in order to evaluate the effect of GA on electrochemical behaviour of CA. Curves a-g in Figure 4C showed the correspondent CV's. The experimental anodic peak current of the mixed solutions varied with GA concentration, but the corresponding slope was smaller than that expected for the theoretical anodic peak current variation (the sum of the peak currents of the two separate voltammograms), that is:

$I_{p,a}$ (CA+GA) $_{experimental}$ = 0,0029 [GA] + 0,5024; R^2 = 0,998,

and

$I_{p,a}$ (CA+GA) $_{theoretical}$ = 0,0038 [GA] + 0,5794; R^2 = 0,9895.

The experimental findings showed that, when the GA concentration increased, I_{pa} (CA+GA) approached $I_{p,a}$ (GA) indicating the total CA disappearance, as shown on Figure 4D. On the reverse scan, the $I_{p,c}$ (CA+GA) $_{experimental}$ results proved that the irreversibility of redox system process for the mixture CA+GA increased with GA concentration, that is, the cathodic current values rapidly decreased with GA concentration and approached zero for 125 µM GA.

The present results have demonstrated that the products of the oxidation and subsequent chemical reactions of GA in the mixture CA+GA, rapidly removed the generated products of the oxidation of CA and, moreover reduced the CA concentration at the electrode surface as above mentioned.

4. CONCLUSIONS

The initially observed GA' negative synergetic effect on biosensing amperometric detection may be attributed to GA' inhibitory effect on the TvLac activity. In fact, the results presented in this work have demonstrated that GA reduces the TvLac-activity in free and immobilised PES/TvLac. Additionally, it has been demonstrated that GA' oxidation and subsequent chemical reaction products, interfered with CA oxidation products, contributing to the effective biosensor signal reduction due to the disappearance of enzymatically oxidised end products.

ACKNOWLEDGEMENTS

D.M.A Gil acknowledges FCT (Fundação para a Ciência e a Tecnologia) for a Ph.D. fellowship (SFRH/BD/34511/2006). The authors acknowledge Financial support from Faculdade de Ciências da Universidade de Lisboa.

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ARTICLE IV

Herbal infusions bioelectrochemical polyphenolic index.

Green tea - the gallic acid interference.

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Food Chemistry, *revised*

ABSTRACT

An amperometric biosensor containing immobilised laccase from *Trametes versicolor* was used for the quantification of phenolic compounds in herbal infusions and green tea samples, from nine botanical families. The main purpose of this research was to correlate the bioelectrochemical polyphenolic index (BPI) of the samples with the total phenolic content according to the Folin–Ciocalteu spectrophotometric method (TPC), and the total antioxidant activity (TAA) measured by TEAC (Trolox Equivalent Antioxidant Capacity). A strong correlation between BPI, TPC, and TAA was obtained for ten herbal infusion samples. However, when a green tea sample was taken into account, a decrease in the linear correlation coefficient (r^2) from 0.9949 to 0.2599 and 0.5609 to 0.1086 for the relationship between BPI/TPC and BPI/TAA, respectively was observed. Thus, we could conclude that the green tea' matrix affects the laccase-based biosensor response. HPLC-DAD analysis showed the presence of gallic acid only in the green tea sample. The results have indicated that gallic acid inhibited the laccase activity regarding the ABTS oxidation in a concentration-dependent manner.The very strong correlation between BPI/TPC obtained for herbal infusions, allow us to conclude that the laccase-based biosensor, used in this research, provides a valuable tool to obtain a valid estimation of the classical Folin-Ciocalteu index, in an uncomplicated and fast way.

Keywords: Green tea; Herbal infusions; Gallic acid; Polyphenols; Laccase from *Trametes versicolor*; Biosensor.

1. INTRODUCTION

Herbal infusions and green tea are some of the most widely consumed beverages in the world, consisting in a major source of dietary polyphenolic compounds, which are considered the most abundant natural antioxidants (Atoui et al. 2005; Alarcón et al., 2008; Vaquero et al, 2010). Phenolic antioxidants in herbs are mainly composed of phenolic acids and flavonoids (Zheng & Wang, 2001), which have revealed to have health-protecting capacity (Pandey & Rizvi, 2009; Vaquero et al, 2010).

The determination of phenolic compounds is regarded to be of great importance in food quality characterisation. For that purpose, there are a number of different analytical methods available, such as GC, HPLC (Naczk & Shahidi, 2004) and spectrophotometric methods. The Folin-Ciocalteu method is one of the most accepted approaches for the spectrophotometric determination of the total content of plant' food phenolics (Robbins, 2003). However, this method can lead to an overestimation of the total of phenolic compounds due to its poor specificity, since Folin-Ciocalteau reagent reacts with other different reducing nonphenolic substances (Singleton & Rossi, 1965; Beer et al., 2004).

A large number of amperometric biosensors has been seen and proposed as an attractive as alternative analytical methods for the quantification of phenolic compounds (Mello et al., 2005; Abhijith et al., 2007; Girelli et al., 2009; Arecchi et al., 2010) since they are characterised by their inherent specificity, simplicity and fast response, being most of them based on the immobilisation of laccase on different electrode supports (Gamella et al., 2006; Elkaoutit et al. 2008; Ibarra-Escutia et al., 2010; Montereali et al., 2010; Gil & Rebelo, 2010).

As it is well known, the great challenge in the biosensor field is the correlation between the data obtained using biosensors with those achieved by classical methods. One of the aims of this work was to correlate the caffeic acid equivalent of ten different herbal infusion and green tea using our laccase-based biosensor (Júnior & Rebelo, 2008) with the Folin-Ciocalteu index. The other goal was to correlate the biosensor data with the herbal infusion and green tea antioxidant

capacity using the TEAC (Trolox Equivalent Antioxidant Capacity) methodology. In our previous work, we came to the conclusion that gallic acid strongly interferes on laccase - based polyphenolic biosensing. Many reports indicated that gallic acid is the most abundant phenolic acid in tea (Yang et al., 2007; Hu et al., 2009). On the other hand, some research groups on amperometric laccase-based biosensors mentioned to have obtained a low sensitivity for gallic acid (Gamella et al., 2006; Elkaoutit et al. 2008; Montereali et al., 2010). Thus, the present study investigated this potential interference in laccase-based biosensor response using samples of herbal infusion and green tea.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Trametes versicolor laccase (E.C.1.10.3.2, 30.6 U mg $^{-1}$), caffeic acid, gallic acid, trolox (6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid), Folin–Ciocalteu's phenol reagent (FC reagent) were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). C. ABTS (2,2-azinobis(3- ethylbenzothiazoline-6-sulfonic acid)) in the crystallized diammonium salt form was from Roche (Roche Diagnostics GmgH, Mannheim, Germany). Potassium persulfate ($K_2S_2O_8$) purity superior to 99% and HPLC-grade methanol were obtained from Merck (Merk KGaA, Darmstadt, Germany). Polyethersulfone membranes (Ultrabind US450 0.45 µm) were from Pall Gelman (Pall Corp., NY, USA). All other chemical reagents used were of analytical grade. All aqueous solutions were prepared using water purified with a Milli-Q A10 system (Millipore).

2.2. Plant materials and preparation of infusions

The eleven different commercial, pre-packaged, dry herbs were purchased from a supermarket in Lisbon, Portugal (Table 1). One gram of each herb was added to 100 ml of boiling water and the infusion was allowed to cool down for 30 min, filtered through 0.45 µm nylon filter membrane

into a volumetric flask of 100 mL and diluted to the mark with ultrapure water. Herbal infusion solution aliquots of 1 mL volume were frozen until use.

2.3. Determination of total polyphenolic content

2.3.1. Laccase-based biosensor method

Bioelectrochemical polyphenolic index (BPI) in herbal infusions was determined by amperometric biosensor. Laccase-based biosensor preparation and amperometric measurements were performed as described in (Júnior & Rebelo, 2008). Briefly, laccase at 9.8 mg mL^{-1}solution (acetate buffer of pH 4.5) was covalently immobilised on modified PES membranes. The bioelectrode was prepared by attaching the enzyme-PES modified membrane to the electrode base system, with an o-ring. The bioelectrode was immersed in 5 mL of 0.033 M tartarate buffer solution pH 3.5, the potential was applied and, when the base current reached a stable value, injections of standard (50 µL) (caffeic acid 0.5 mM in tartrate buffer pH 3.5) were done, under magnetic stirring, at 300 rpm. The experiments were made at a constant potential (100 mV versus Ag/AgCl). The current response corresponding to the reduction of enzymatically produced *o*-quinone was recorded as function of time. The biosensor was used to measure the relevant cumulative effect of the polyphenolic content of herbal infusions. Herbal infusion samples (200 µL) were injected into 5 mL of the buffer (tartarate at pH 3.5), following the same procedure as for the standard. The results were expressed as milligrams of Caffeic Acid Equivalents (CAE) per g of dry weight of plant, averaged from triplicates.

2.3.2. Folin–Ciocalteu method

Total phenolic compound content (TPC) in herbal infusions was determined spectrophotometrically, according to the Folin–Ciocalteu (FC) colorimetric assay with the microscale protocol (Waterhouse, 2001), which is based on the method reported in (Singleton & Rossi, 1965), using caffeic acid as a standard. The phenolic content in herbal infusion was evaluated from the generated absorbance value by the interpolation into calibration plot (A =

0.0011 caffeic acid (mg L^{-1}) - 0.004; r^2 = 0.9935). The results (averaged from triplicates) were expressed as milligrams of Caffeic Acid Equivalents (CAE) per g dry weight of plant.

2.4. Determination of antioxidant activity by the ABTS method

Determination of total antioxidant activity (TAA) of herbal infusions was performed by Trolox Equivalent Antioxidant Capacity (TEAC) method, using the ABTS radical cation solution, as described by (Re et al., 1999) with slight modifications (Fernandéz-Pachón et al., 2004). The absorbance of the resulting oxidised solution was compared to that of the calibrated Trolox standard (A= 0.0043 trolox (µM) – 0.0119; r^2 = 0.9966). Results (averaged from triplicates) were expressed in terms of Trolox Equivalent Antioxidant Capacity (TEAC, µmol Trolox equivalents per g dry weight of plant).

2.5. HPLC – DAD analysis

The HPLC analysis was carried out in Liquid Chromatograph Finnigan™ Surveyor® Plus Modular LC System (Thermo-Finnigan, Bremen, Germany), equipped with a LiChroCART® 250-4 LiChrospher® 100 RP-8 (5 µm) column, from Merk (Merk KGaA, Darmstadt, Germany), and Xcalibur software version 1.2 (Thermo-Finnigan, Bremen, Germany). The herbal infusion samples were filtered through 0.2 µm filters, diluted 1:2 (v/v) with water and analysed by HPLC by injecting 25 µl and using a linear gradient with a constant flow of 0.800 mL min^{-1}composed of solution A (0.05% trifluoroacetic acid), solution B (methanol) as following: 0 min, 90% A; 40 min 30% A; 45 min, 30% A. The detection was carried out between 200 and 600 nm with a diode array detector. The gallic acid standards were run under the same conditions. For the quantification of gallic acid in the herbal infusion samples, the linear regression equation for gallic acid standard was obtained by plotting the concentration of standard compound injected against the peak area (Area = 98426 gallic acid (mg L^{-1}) + 11948; r^2 = 0.9988).

2.6. Inhibition studies

The spectrophotometric assays were carried out on a UV-Vis double beam Shimadzu UV-1603 spectrometer (Shimadzu Corp., Kyoto, Japan) connected to a PC (UVPC® Personal Spectroscopy Software version 3.7 (Shimadzu Corp., Kyoto, Japan)). The final volume of all enzymatic reactions mixtures was 1.3 mL, in a 3 mL, 1cm width, UV-Vis cuvette. The reaction mixture consisted of 1000 µL sodium acetate buffer (10 mM, pH 4.5), 0-200 µL of inhibitor solution [(gallic acid stock solution (1.47 mg L^{-1}) or green tea (diluted 1:10 (v/v))], 100 µL enzyme solution (0.015 mg mL^{-1}) and 100µL substrate (0.5 mM ABTS, final concentration in the cuvette). The reaction was run at 25ºC and started by the addition of substrate solution to the enzyme solution. The ABTS oxidation rate was determined spectrophotometrically (420 nm) by monitoring the coloured oxidation products for 2 min. A control test, in which the inhibitor compound was replaced by buffer, was conducted in parallel. The inhibition percentage was calculated by means of the formula: $I(\%) = 100 - (V_{i,sample} / V_{i,control}) \times 100$; where, I: inhibition (%), $V_{i,sample}$: initial oxidation rate (AU min^{-1}), and $V_{i,\ control}$: initial oxidation rate in control test (AU min^{-1}). The IC_{50} values were obtained from the plot of the logarithm of inhibitor concentrations against the percent inhibition of the enzyme activities.Tests were carried out in triplicate and a blank with sodium acetate buffer instead of enzyme solution was undertaken.

2.7. Statistical analysis

All measurements were carried out in triplicate and the results were statistically analysed using the Microsoft Excel software version 2007 (Microsoft Corporation, N.Y, USA) to determine the average value and standard error. Correlation analysis was also performed using the same statistical package. Differences were analysed with single factor one-way ANOVA, followed by a post hoc Tukey test of multiple mean comparisons. Probability values lower than 0.05 were considered statistically significant. Analyses were performed with Sigmaplot-Systat software version 11 (Systat Software Inc., Chicago, USA).

3. RESULTS AND DISCUSSION

The herbal infusions and tea samples selected were diverse regarding type, parts used and morphology. They comprised leaves, flowers, seeds, bark, and stems of 10 species belonging to 10 genera and 9 families (Table 1).

Table 1. Botanical characteristics and major phenolic compounds of the 10 herbs studied.

Name	Family	Plant part used	Major phenolic compounds
Green tea (*Camellia sinensis)*	*Theacea*	Leaves	epigallocatechin gallate, gallocatechin gallate (Peng et al.,2008; Yang , Hwang & Lin, 2007)
Fennel (*Foeniculum vulgare)*	*Apiaceae*	Seeds	Chlorogenic acid, quercetin-3-O-*β*-D-glucuronide, *p*-anisaldehyde and *trans*-anethole (Bilia et al., 2000)
Pau d' arco *(Tecoma violaea)*	*Bignoniaceae*	Bark	quercetin, apigenin, cyandin-3-rutoside, and ferulic acid (Cai et al., 2004)
Linden *(Tília vulgaris)*	*Tiliaceae*	Aerial parts / flowers	Quercetin, kaempferol (Horžić et al., 2009)
Horsetail *(Equisetum arvense)*	*Equisetaceae*	Aerial parts	di-*E*-caffeoyl-*meso*-tartaric acid, caffeic acid and protocatechuic acid (Mimica-Dukic et al., 2008)
St. John's wort *(Hypericum perforatum)*	*Clusiaceae*	Aerial parts	Isoquercitrin , chlorogenic acid (Wach et al., 2007; Hernandez et al., 2010)
Gorse *(Pterospartum tridentatum)*	*Fabaceae*	Flower heads	Isoquercitrin, isoflavones and flavonol glucoside (Vitor et al., 2004)
Dandelion *(Taraxacum officinale)*	*Asteraceae*	Aerial parts	caffeic acid, chlorogenic acid, and luteolin-7-glucoside (Hu & Kitts, 2005)
Peppermint *(Mentha piperita)*	*Lamiaceae*	Aerial parts	Rosmarinic acid and Eriocitrin (Capecka, Mareczek, & Leja, 2005; Lee, 2010)
Lemon balm *(Melissa officinalis)*	*Lamiaceae*	Aerial parts	Rosmarinic acid (Capecka, Mareczek, & Leja, 2005; Lee, 2010)

The total antioxidant activity (TAA), the caffeic acid equivalent measured by the laccase- based biosensor (BPI), the total phenolic content according to the Folin–Ciocalteu spectrophotometric method (TPC) of 10 herbal infusions and tea samples were analysed and compared. The samples were arranged from the lowest to the highest bioelectrochemical polyphenolic index, and the results of TAA, BPI and TPC shown on Table 2.

Despite the difficulty in directly comparing the results from different studies due to the difference in the experimental protocols, standards and units in which the results were expressed, a comparison study of the phenolic contents and total antioxidant values with literature data was undertaken, when possible.

3.1. Antioxidant activities of herbal infusions and tea samples

The ABTS assay for estimating antioxidant activity of herbal infusions was chosen among other similar methods for being highly directed to water-based solutions (Fernández-Pachón et al., 2004; Samaniego-Sánchez et al., 2010). Moreover, it displays a better correlation coefficient between antioxidant activity and polyphenolic content, when compared e.g. with DPPH method, as it was recognized in the literature (Samaniego-Sánchez et al., 2007).

The free-radical scavenging activity of different herbal infusions was estimated by the ABTS method. The total antioxidant capacity (TEAC) values express the relative ability of hydrogen- or electron-donating antioxidants of an herbal infusion sample to scavenge the ABTS radical cation compared to that of trolox, a water soluble analogue of α-tocopherol. The TEAC value was obtained by interpolating the decrease in absorbance of a corresponding diluted herbal infusion sample on the calibration curve, obtaining a concentration of trolox. Thus, the TEAC value of an herbal infusion expresses the concentration of trolox solution whose antioxidant activity was the same as that of the herbal infusion. The Total antioxidant activities (TAA) of herbal infusions are shown on Table 2. The TAA ranged from 31.3 to 372.5 TEAC, μmol g^{-1}dw

in fennel and green tea, respectively (Table 2). Analysis of variance of means showed that there was no significant statistical difference among fennel, horsetail, and St. John's wort (Table 2).

3.2. Polyphenolic contents of herbal infusions and tea samples

The chemical composition of the herbal infusions and tea samples used during this analytical work was reported in previous works and the main components of these plant extracts were found to be phenolic compounds (Table 1). The amount of total phenolics determined by us, using our biosensor and the Folin–Ciocalteu method, varied in different herbal infusions and ranged from 0.00 to 10.92CAE, mg g^{-1} dw and from 13.2 to 110.0 CAE, mg g^{-1} dw, respectively. The highest total phenolic levels measured by both biosensor and Folin–Ciocalteu methods were detected in lemon balm. Green tea had the highest phenolic level when determined by the Folin–Ciocalteu method and the lowest when the biosensor was used. Low levels measured by biosensor were also found in fennel, pau d´arco, linden, horsetail and St. Jonh´s wort herbal infusions; while the method Folin – Ciocalteu gave the lowest phenolic levels for fennel, pau d´arco, linden, horsetail and St. Jonh´s wort (Table 2).

Table 2. Bioelectrochemical polyphenolic index (BPI), total phenolic content (TPC) and total antioxidant activity (TAA) of herbal infusion and tea samples.

Herbal Infusion and tea samples	BPI (CAE, mg g-1dw) [A,B]	TPC (CAE, mg g-1dw) [A,B]	TAA (TEAC, µmol g-1dw)[A,C]
Green tea	0.00 ± 0.00 [a]	110.0 ± 2.4 [e]	372.5 ± 5.7 [h]
Fennel	0.35 ± 0.12 [a]	13.2 ± 0.1 [a]	31.3 ± 0.6 [a]
Pau d' arco	0.38 ± 0.03 [a]	15.3 ± 0.5 [a]	55.0 ± 2.7 [b]
Linden	0.76 ± 0.08 [a]	19.1 ± 1.3 [a]	84.7 ± 1.4 [c]
Horsetail	0.87 ± 0.04 [a]	17.3 ± 0.8 [a]	39.0 ± 4.0 [a]
St. John's wort	1.23 ± 0.09 [a]	22.2 ± 0.4 [a]	36.7 ± 1.1 [a]
Gorse	3.94 ± 0.23 [b]	44.1 ± 0.6 [b]	106.6 ± 0.7 [d]
Dandelion	4.42 ± 0.33 [b]	40.9 ± 1.1 [b]	141.2 ± 0.7 [e]
Peppermint	7.62 ± 0.36 [c]	66.4 ± 1.3 [c]	332.3 ± 1.4 [g]
Lemon balm	10.92 ± 0.25 [d]	91.2 ± 1.4 [d]	158.4 ± 4.7 [f]

[A] Each value is the mean of triplicates ± standard deviation (SD).

[B] Expressed as mg caffeic acid equivalents (CAE) per g of dry weight of plant (dw).

[C] Expressed as trolox equivalent antioxidant capacity (TEAC) µmol per g dry weight of plant (dw).

Numbers with different letters within the same column are significantly different ($p \leq 0.05$).

As can be seen, there are a large difference between the obtained Folin–Ciocalteu value and bioelectrochemical polyphenolic index (BPI). Similar results have been reported in the literature (Gamella et al. 2006; Elkaoutit et al. 2008; Ibarra-Escutia et al., 2010). These authors also found that the BPI values obtained by their biosensor were much lower than those obtained by the Folin-Ciocalteu method. Actually, the assay based on Folin-Ciocalteu reagent and commonly known as the total phenolic assay measures a sample's reducing capacity, and therefore the obtained results should be considered as the content of all reducing substances in the herbal infusion, and not considering exclusively the phenolic content (Wang et al., 2010).

Furthermore, the BPI values depend on the standard phenolic compound taken as biosensing reference (Gamella et al., 2006; Gil & Rebelo, 2010).

3.3. Relationship among BPI, TPC and TAA

A great ongoing challenge in biosensor development is the correlation between the data obtained using biosensors and those achieved by classical analytical methods. For this purpose, the data obtained with our laccase-based biosensor was correlated with the values determined by the Folin- Ciocalteu method, which is the most widely used method for total polyphenolic content determination in food chemistry.

As it can be seen on Table 3, there was a very strong linear correlation between BPI values and TPC values, within nine studied botanical families. However, the green tea sample was not taken into account for the correlation analysis, since the simple removal of that sample improved the coefficient of determination (r^2) from 0.2599 to 0.9949 (Table3). The good correlation obtained by excluding the green tea values allows the use of laccase-based biosensor for the estimation of the index of polyphenolic compounds in herbal infusions.

Some studies (Wojdyło, Oszmiański & Czemery, 2007, Liu et al., 2009; Horžić et al., 2009) have demonstrated a good linear correlation between the content of total phenolic compounds and their antioxidant capacity, while others show weak or inexistent linear correlation (Kähköne et al., 1999; Ivanova et al., 2005; Capecka, Mareczek & Leja, 2005). In our study it should be emphasized that, when the green tea samples was not taken into account, there was a moderate and similar linear relationship between the measured values, TAA/BPI (r^2 = 0.5609) and TAA/TPC (r^2 = 0.5468). The TAA/TPC coefficient of determination suggests that approximately 55 % of the antioxidant capacity of samples results from the contribution of phenolic compounds. The moderate correlation between antioxidant activity and total phenolic contents could be related to the presence of non-phenolic compounds (other antioxidant secondary metabolites, such as volatile oils, carotenoids, and vitamins, among others),

antagonistic or synergetic interactions between constituents and also distinct antioxidant activities of individual phenolics (Singleton & Rossi, 1965; Beer et al., 2004; Atoui et al., 2005; Capecka, Mareczek & Leja, 2005).

However, the simple addition of green tea sample, which is known to possess a high antioxidant capacity, increased, as expected, the linear relationship between TAA and TPC (r^2 = 0.7370) and decreased drastically the TAA/BPI coefficient of determination (r^2 = 0.1086) (Table 3).

These results led us to the conclusion that green tea affects the biosensor performance. Thus, it is of relevant importance to evaluate possible interferences of green tea constituents, on laccase biosensor performance.

3.4. Evaluation of interferences on biosensor response

3.4.1. Recovery studies

In order to evaluate the matrix (herbal infusions) effect on the lacasse-based biosensor performance, recovery studies were performed. The experiments involved a recovery of a known quantity of caffeic acid (0.90 mg L^{-1}) standard solution, which was added to the herbal infusion sample at the proportion 1:26 (v/v). The percentage recovery values were calculated by comparing the biosensor response (ΔI (nA)), obtained for the known concentration of caffeic acid with and without the addition of herbal infusion sample. If the recovery result of green tea was not taken into account, the caffeic acid recovery values, reported on Table 4, which ranged between 94% to 117% , indicated that the laccase-based biosensor could be very well applied in the analysis of polyphenolic content in that samples, without significant influence of the matrix. However, it can be clearly observed from the results (Table 4) that the green tea matrix did not allow the recovery of the caffeic acid. Thus, the obtained result suggests that green tea matrix presents a huge interference effect on the biosensor polyphenolic' analysis.

3.4.2. Analysis of phenolic compounds in herbal infusions and green tea using HPLC-DAD

The obtained results emphasized the importance of comparing individual phenolic compounds of the green tea with each one of those from herbal infusion. In previous works, we have demonstrated that gallic acid strongly interferes on polyphenolic amperometric biosensing using laccase from *Trametes versicolor*. Thus, qualitative analysis was carried out using HPLC-DAD, in order to investigate the presence of gallic acid in herbal infusions.

As it is well known, different phenolic compounds normally possess specific chromatographic behavior and UV-vis spectral characteristics. The identification of gallic acid was based on a comparison of retention time and spectral matching. Chromatographic profiles of gallic acid standard and the studied herbal infusions, obtained under identical analysis conditions, are presented on Figure1A. Gallic acid showed a retention time, t_r, of 7.89 min, with a maxima UV absorbance at 217 and 272 nm, as shown on Figure1A, B. The herbal infusions chromatograms indicated that only green tea had a peak with the same gallic acid retention time, and presented the same UV absorption bands as compared to gallic acid standard. The horsetail chromatographic profile showed a peak with a retention time of 7.98 min. According to UV spectrum that peak did not match the gallic acid UV spectrum, since it presented only one UV absorption band at 217 nm (Figure 1B.). Thus, the comparative gallic acid standard – herbal infusions analysis unambiguously demonstrated the presence of gallic acid only in green tea.

Aiming to estimate the concentration of gallic acid in green tea, in order to prepare gallic acid solution that would be used for comparative purposes on inhibition studies, the quantitative evaluation of gallic acid in green tea was performed. That was done by six-point regression curve, plotting peak areas of gallic acid against applied concentration of gallic acid (r^2 = 0.9988) in the range 0 to 50 mg mL^{-1}. The estimated concentration of gallic acid present in green tea was obtained by triplicate and the result was 36.2 ± 0.5 mg L^{-1} (3.62 ± 0.05 mg g^{-1}dw). Our result agreed with those reported by Yang *et al*., 2007. From this result, we found that the

content of gallic acid in studied green tea represents 3.29 % of the total polyphenolic content estimated by Folin –Ciocalteu method.

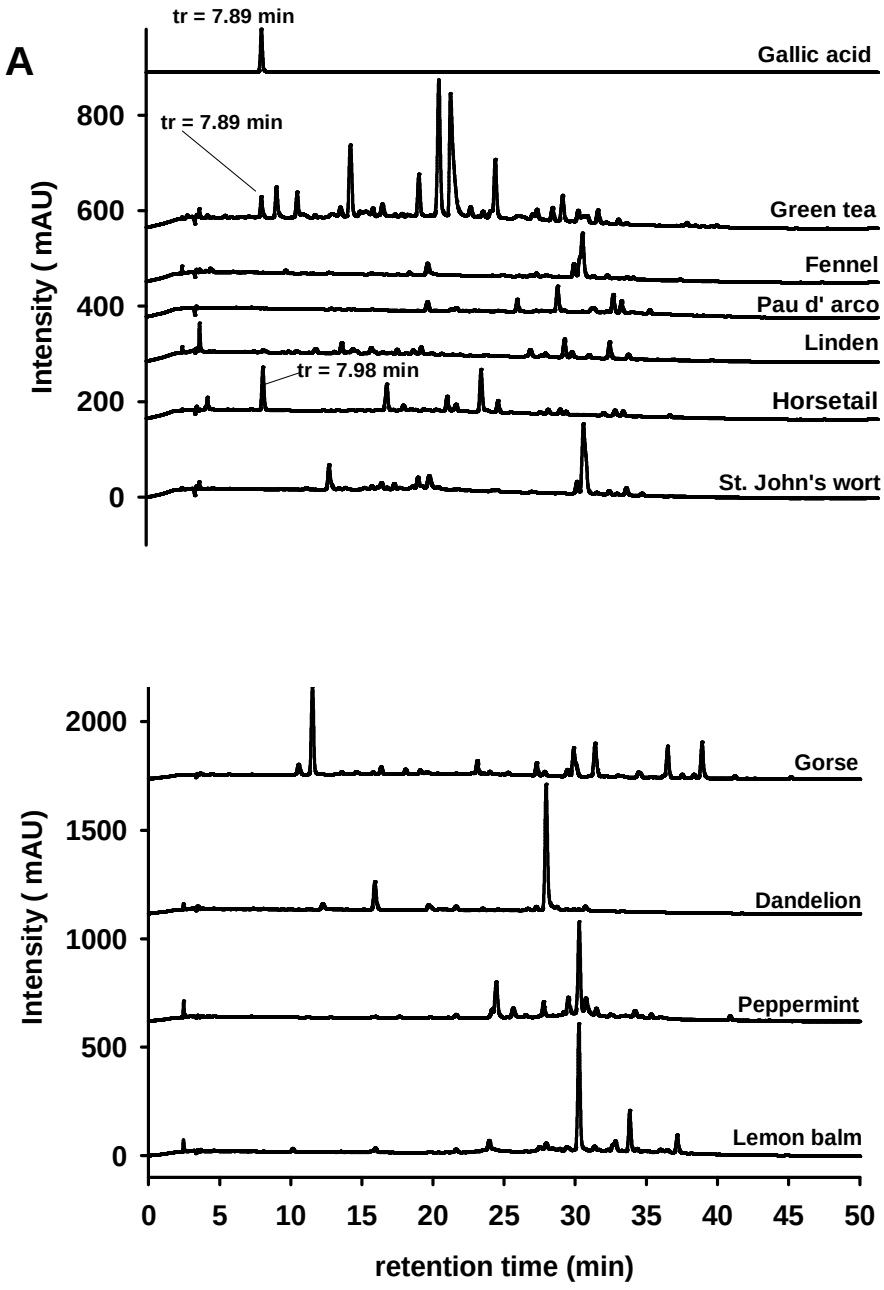

Figure 1. A - HPLC chromatograms of gallic acid standard at 40 mg L^{-1} and studied herbal infusions and green tea diluted 1:2.

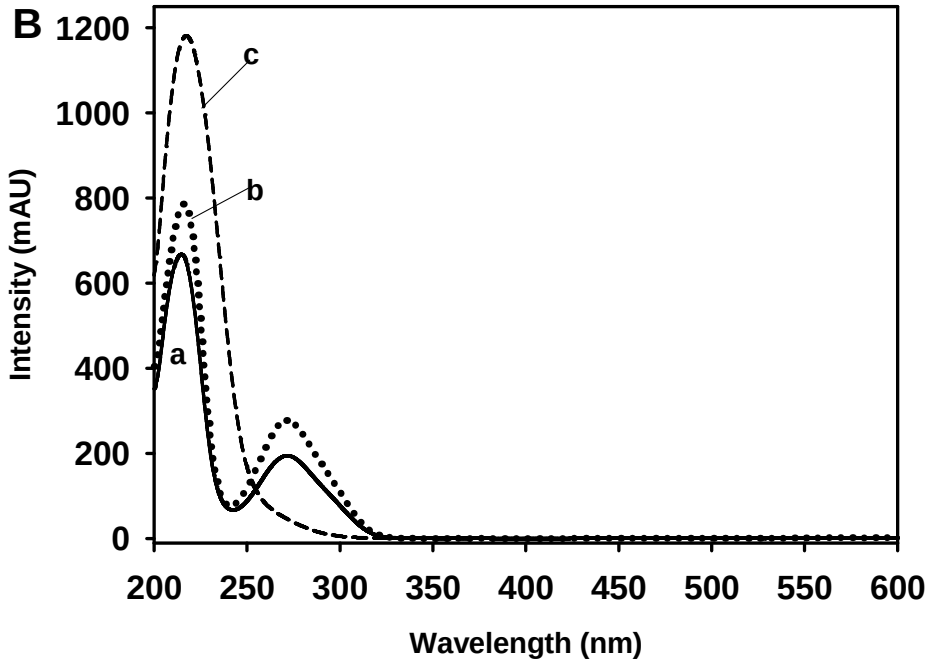

Figure 1. B - UV spectrum of a) gallic acid standard (t_r = 7.89 min); b) peak retention (t_r = 7.89 min) from green tea and, c) peak retention (t_r = 7.98 min) from horsetail infusion.

3.4.3. Studies of the influence of gallic acid on the activity of the enzyme laccase

The presence of gallic acid and the correspondent influence on free laccase action on oxidative reaction of ABTS was studied using a spectrophotometric method. The relationship between residual enzyme activity and the concentration of gallic acid is shown on Figure 2. It was, therefore, evidenced that gallic acid inhibited the activity of free laccase with respect to ABTS oxidation. The results showed that the inhibition was concentration dependent. In fact, the residual laccase activity decreased as the concentration of gallic acid increased. The half-maximal inhibitory concentration (IC_{50}) value was obtained plotting the logarithm of inhibitor concentrations against the percent inhibition of the enzyme activities. The IC_{50} for green tea was 0.12 ± 0.03 mg L^{-1}.We could conclude that the obtained IC_{50} value corresponded only to gallic acid' inhibition, since the corresponding half-maximal inhibitory concentration value for gallic acid' standard solution was 0.16± 0.01 mg L^{-1}. Thus, the obtained results indicated that gallic acid presented an interference effect on the biosensor biological recognition component.

4. CONCLUSIONS

The findings presented in this work have demonstrated that herbal infusions tea samples have a bioelectrochemical polyphenolic index (BPI) that strongly correlated with the total phenolic content according to the Folin–Ciocalteu spectrophotometric method (TPC): r^2 equaled 0.9949, and a good correlation with the total antioxidant activity (TAA) measured by TEAC was obtained: r^2 equaled 0.5609. However, when the green tea sample was taken into account, the BPI of the samples would weakly correlated with the total TPC, and TAA. The coefficient of determination being 0.2599 and 0.1086 for correlation between BPI/TPC and BPI/TAA, respectively. Thus, samples containing gallic acid (e.g. green tea) which reduces / inhibits *Trametes versicolor* laccase activity, need further studies when polyphenol oxidase based biosensors are used.

The high correlation results obtained during this analytical study on the subject of herbal infusions, from eight botanical families, allowed us to conclude that the laccase-based biosensor used in this research is a good method for the estimation of the index of polyphenolic compounds of those herbal infusions.

ACKNOWLEDGEMENTS

Dulce M.A. Gil and Pedro L.V. Falé acknowledge FCT (Fundação para a Ciência e a Tecnologia) for the Ph.D. Grants SFRH/BD/34511/2006 and SFRH/BD/37547/2007, respectively. The authors acknowledge Financial support from Faculdade de Ciências da Universidade de Lisboa

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CHAPTER 4

GENERAL CONCLUSIONS

The main goal of this thesis was to optimise the applicability of a new methodology to evaluate the antioxidant activity of wines and teas, using a laccase-based biosensor in relation to their polyphenolic content, and also to compare the corresponding measured values (the bioelectrochemical polyphenol index (BPI)) to those of other methods, such as total phenolic content (TPC) using Folin–Ciocalteu method, and total antioxidant activity (TAA) using ABTS assay. Interferences studies on polyphenolic amperometric biosensing were performed for metabissulfite and gallic acid (GA). The biosensor parameters and optimisation of suitable analytical conditions for measurements were previously performed by our group.

The results obtained by means of the studies discussed in this thesis showed that:

A good correlation was observed between the antioxidant properties (TAA) of the studied wines (9 red, 5 white and 3 rosé wines), which was determined by the ABTS assay and also the BPI with the biosensor at pH 3.5, taking caffeic acid as the reference solution. The linear relationship between TAA and BPI enabled the calculation of the antioxidant activity of red, rosé and white wines, from its caffeic acid equivalent content, evaluated by the present biosensor. Our laccase-based biosensor methodology presents advantages when compared to the methods referred above. In fact, it is straightforward to prepare due to the rapid immobilisation of the enzyme on the derivatized polyethersulfone membranes. Moreover, standards and measurements were at pH 3.5, which is a value close to the average pH of wines. The present biosensor, with a disposable membrane, has potential application in the food industry as a bioelectrochemical polyphenol index. This application follows the trend to exploit biosensor technology in areas other than medical diagnostics, where commercial glucose disposable biosensors are being currently used.

Sodium metabisulfite was found to influence both measurements taken with laccase-based biosensors and the Folin-Ciocalteu spectrophotometric method.

It was also observed that there was a GA' negative synergetic effect on biosensing amperometric detection, which could be attributed to GA' inhibitory effect on the TvLac activity. In fact, the results presented in this work have demonstrated that GA reduces the TvLac-activity in free and immobilised PES/TvLac. Additionally, it has been demonstrated that GA' oxidation and its subsequent chemical reaction products have interfered with CA oxidation products, contributing to a reduction of the effective biosensor signal, due to the disappearance of enzymatically oxidised end products.

The findings presented in this work have demonstrated that herbal infusions and tea samples have a bioelectrochemical polyphenolic index (BPI) that strongly correlates with the total phenolic content according to the Folin–Ciocalteu spectrophotometric method (TPC): r^2 equaled 0.9949, being that a good correlation with the total antioxidant activity (TAA) measured by TEAC was also obtained: r^2 equaled 0.5609. However, when the green tea sample was taken into account, the BPI of the samples would weakly correlated with the total TPC, and TAA. The coefficient of determination being 0.2599 and 0.1086 for correlation between BPI/TPC and BPI/TAA, respectively. Thus, samples containing gallic acid (*e.g*. green tea), which reduces/inhibits *Trametes versicolor* laccase activity, need further studies when polyphenol oxidase based biosensors are used.

The very strong correlation achieved between BPI/TPC for herbal infusions, allow us to conclude that the laccase-based biosensor, used in this research, provides a valuable tool to obtain a valid estimation of the classical Folin-Ciocalteu index, in an uncomplicated and fast way.

So, we support the recommendation that results obtained in wine and teas with laccase-based biosensors and Folin-Ciocalteu spectrophotometric method should be reported as "caffeic acid equivalent" and more work should be done in order to quantify and/or avoid the above mentioned interferent substances.

Lisboa, 29 de Abril de 2011

Dulce Martins de Albuquerque Gil